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SPERMATOGENESIS OF THE PACIFIC COAST EDIBLE CRAB, *CANCER MAGISTER* DANA.

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1. Introductory Remarks.

The Decapoda have always been of absorbing interest to the cytologist and considerable has been written concerning their germ cells. In a previous publication ('14), I have given a review of the important literature on the subject, and by referring to this article it becomes apparent that practically all the investigations have been confined to the male reproductive elements of the Macrura and Anomura. Virtually nothing has been done with the Brachyura; in fact there isn't a single complete spermatogenesis of a crab known. Carnoy ('85) observed and pictured a few of the early proliferation stages in the spermatogenesis of *Carcinus menas*, while Binford ('13) hastily described the spermatogenesis of the Atlantic coast edible crab, *Menippe mercenaria*. None of these investigators, however, have given details regarding the interesting processes of synapsis, reduction, chromosome numbers and the like.

During the past few years the writer has been gathering testicular material of numerous crabs which occur along Puget Sound, for the purpose of studying their spermatogenesis, and in the following pages the spermatogenesis of one of these forms, namely, *Cancer magister*, is described.

2. Material and Methods.

As already mentioned, the material from which these studies were made consisted of the testis of the common edible crab of the Pacific coast, *Cancer magister*. This Brachyuran is widely distributed along Puget Sound, and an abundance of material was available for study. Most of this was gathered in the vicinity of the Puget Sound Biological Station, Friday Harbor, Washington, during the summers of 1915 and 1916. In the latter part of June and the early portion of July, the testis of *Cancer magister* is in the best condition for the study of spermatogenesis. At these times every stage in the spermatogenesis process may be found within the male gonads.

The author worked almost exclusively with smears of the testicular cells. Sectioned material was also used for comparison and checking up results. The smears, however, were of greatest service and virtually all the deductions and illustrations were made from them. The manner in which the smears were prepared was as follows. Small pieces of the fresh testis were quickly mashed between two slides and immediately fixed in Bouin's fluid for about ten minutes. The slides were then washed in water until all traces of the yellowish picric acid were removed. They were next stained by the iron-alum haematoxylin method, with a counterstain of acid-fuchsin. Finally, they were run up in the usual manner through the alcohols into xylol and when fully cleared were mounted under cover-glasses with Canada balsam. Some of these preparations were unsurpassed for details regarding synapsis, chromosome numbers, spermatid transformations, and steps in the opening of the spermatozoa. With the exception of Figs. 1–15 and Figs. 67–69, all the drawings in the accompanying plates are from smear preparations. Figs. 1–15 are from sections, while Figs. 67–69 are from living spermatozoa as viewed in the crab's body fluids.

The material for sectioning was cut into small pieces and fixed with numerous fluids. The best fixatives, however, were found to be Flemming's strong and the Meves-Duesberg modified Flemming. The sections were cut 5μ in thickness and then stained by either the iron-alum hæmatoxylin method with a counterstain of acid-fuchsin or by the safranin, gentian-violet method.

The living spermatozoa were studied under the oil-immersion lenses in various fluids, such as the body fluids of the crab, sea water, in isotonic and hypotonic solutions of various salts (NaCl, KCl, $CaCl_2$, $NaNO_3$ and KNO_3), and in distilled water. By fixing the spermatozoa on the slide with either osmic acid fumes or Bouin's fluid, these structures could then be stained in iron-hæmatoxylin and acid-fuchsin and all the stages in their explosion could subsequently be studied.

3. Description of Testis.

The testis of *Cancer magister* has already been described elsewhere (Fasten, '15). It is a bilobed organ lying in the cephalothoracic region, below the cardiac chamber and above the digestive glands. Each lobe is profusely tubular and runs laterally along the stomach. During June and July these testicular lobes are prominently developed and fill up a large part of the upper cavity of the cephalothorax. Below the anterior portion of the heart the two lobes of the testis unite, and from this junction point two stoutly convoluted tubes, the vasa deferentia, originate and run posteriorly to the base of the fifth pair of walking legs where they open to the outside.

During the latter part of June and the early part of July the tubules in the outer and middle regions of each testicular lobe are usually undergoing rapid proliferation. Some of them are filled with spermatogonia, while others contain growth stages and still others show primary and secondary spermatocyte divisions. Also, many of the tubules show spermatids transforming into spermatozoa, although the mature spermatozoa are mainly confined to the tubules located near the inner or median portion of the testicular lobe as well as in the spermatophores of the vasa deferentia.

When sections of the tubules are examined, many of them are observed to be constructed on the same plan as those of *Menippe mercenaria* which were described and pictured by Binford ('13). Two or three well-defined zones may oftentimes be distinguished in one tubule. For instance, at one pole in the sectioned tubule there might be a thin layer of spermatogonia and the rest of the tubule might contain either transforming spermatids, or mature spermatozoa, or primary spermatocyte divisions, or those of the secondary spermatocyte stage, or even growth stages. When there are three zones in the tubule these are often made up of (1) a thin region of spermatogonia at one end, (2) a middle portion filled with transforming spermatids, and (3) a region of mature spermatozoa filling in the opposite pole.

The mature spermatozoa, when they enter the vasa deferentia, are surrounded by pouches known as spermatophores (Fig. 67). These spermatophores are formed by the secretions of the inner layer of epithelium which lines the vas deferent tube. For a more detailed description of these structures see Fasten, '17. During copulation the spermatophores are discharged from the vasa deferentia of the male and are deposited within the seminal receptacle of the female, where they remain dormant until the ova are mature for fertilization.

4. SPERMATOGENESIS.

A. *Spermatogonial Stages.*

The spermatogonial cells generally line one end of the tubule. They are fairly large cells, with distinct cytoplasmic outlines and prominent nuclei. In *Cancer magister*, primary and secondary spermatogonial stages can be distinguished (Figs. 1–8). Of the two, the primary spermatogonium (Fig. 1) is somewhat larger in size, and from it by division (Figs. 2–7), is derived the secondary spermatogonium (Fig. 8).

The resting primary spermatogonium (Fig. 1) contains numerous large chromatin clumps within the nucleus. In some instances a number of linin strands could also be distinguished. The cytoplasm is more or less uniform throughout, but in a few cases large heavily staining masses, surrounded by clear outlines (Fig. 2), could be observed within it. A centrosome is almost always discernible.

When the cell begins to divide the large chromatin clumps of the nucleus undergo fragmentation (Fig. 2). This process continues until the chromatin becomes organized into a great number of heavily staining elliptical or oval structures distributed throughout the nucleus. The spermatogonium at this stage presents the picture shown in Fig. 3. Counts of these chromatin clumps were attempted, but they were found to vary considerably, ranging everywhere from forty to sixty-five. Binford ('13) has made similar observations in *Menippe mercenaria* and in the American crayfish *Cambarus virilis*, I ('14) have found the same sequence of events. Binford regards these chromatin clumps as the chromosomes which enter the equatorial plate of the spermatogonial metaphase. While these through a further fragmentation, undoubtedly form the ultimate number of chromosomes found during the metaphase stage of the spermatogonial division, yet I am inclined to the view that many of these chromatin clumps represent more than single chromosomes. This conclusion was arrived at after examination of numerous polar views of the chromosomes in metaphase plates of primary spermatogonia, in which over one hundred chromosomes could be counted. Furthermore, these chromosomes were smaller than the chromatin clumps shown in Fig. 3.

The nuclear wall surrounding the chromatin clumps soon breaks down and the cell enters the metaphase stage (Fig. 4). The chromosomes in the equatorial plate are dumb-bell shaped and the spindle fibers are rather delicate in appearance. The centrosomes can be easily distinguished at opposite poles. In polar views of sections of the equatorial plate, the chromosomes are observed to be distributed throughout the whole plane of the equator. They are small and spherical in appearance, and are so numerous that their exact number could not be determined. As already mentioned above, in many instances over one hundred of them could be counted. But on the assumption that there are twice the number of spermatogonial chromosomes as are found in the reduction division, there must be one hundred and twenty chromosomes in the spermatogonial stages of *Cancer magister.*

The anaphase (Fig. 5), and telephase (Figs. 6 and 7) stages follow each other quickly, thus dividing the cell into secondary spermatogonia (Fig. 8). These are somewhat smaller than the primary spermatogonia (compare Figs. 1 and 8), and in the resting condition (Fig. 8), the chromatin of their nuclei stains much more heavily. Otherwise, the secondary spermatogonia resemble the primary ones markedly and their division proceeds in exactly the same fashion. The ultimate divisions of the secondary spermatogonia produce the resting primary spermatocytes (Fig. 16).

In many spermatogonial strips of the tubules, some of the cells were found to be undergoing degeneration. In such cases, the cells lose their distinctness of outline and their nuclei come to lie in a syncitial mass of protoplasm. In many instances, the nuclei resemble those of the spermatogonia (compare Fig. 9 with Fig. 1); in others (Figs. 10–12), the nuclei become transformed into very large irregular structures with pseudopodia-like projections. These cells are the so-called "nutritive cells" and they may be best studied in tubules where mature spermatozoa are found. In *Cambarus virilis*, I ('14) have noticed similar cells.

The nutritive cells (Figs. 9–15) are very interesting structures. Their nuclei contain heavily staining chromatin masses, while the cytoplasm possesses numerous fat globules which stain intensely black with osmic acid. Many of the earlier investigators on the Decapoda such as Grobben ('78), Gilson ('86), and Herrmann ('90) have claimed that the spermatogonia are derived from the nutritive cells. On the other hand, St. George ('92), and Keppen ('06) claim the opposite, that the nutritive cells are derived from a transformation of the spermatogonia. This last-mentioned condition seems to be the case in *Cancer magister.*

When sections of the nutritive cells are examined, numerous stages like those seen in Figs. 13–15 may be observed, which strongly suggest amitosis. The fact is that the earlier workers on the Decapoda all claim such division in the nutritive cells. However, a careful study of the nuclei shows many of them to be ambœoid in appearance (Figs. 10–12), and this being the case, it is entirely possible to derive the stages represented in Figs. 13–15 from sections through such cells as are shown in Figs. 10–12. It is, therefore, very difficult as well as dangerous to come to any certain conclusions concerning amitosis from sectioned material.

B. *Primary Spermatocyte Stage.*

This stage follows the spermatogonial divisions. After a period of growth and synapsis, reduction occurs. During the growth period two definite spherical bodies, surrounded by clear spaces, make their appearance in the cytoplasm. These are the so-called chromatoid bodies, and they appear to be similar to the same structures which I ('14) have previously described in *Cambarus virilis.*

(*a*) *Growth Period.*—This period includes the preparatory stages, synapsis and tetrad formation. During the early prophases the chromatin in the nucleus of the resting primary spermatocyte (Fig. 16), consists first of a few large, heavily staining clumps, but these soon undergo a fragmentation process, whereby smaller chromatin masses (Fig. 17) are produced. In a few cells, linin threads which were rather indistinct and granular in appearance were observed. Sometimes large chromatoidal masses like those shown in Fig. 17 were seen within the cytoplasm.

The chromatin of the nucleus breaks up into still smaller structures and these then begin to weave out into thin leptotene threads (Fig. 18). At the same time the cell increases somewhat in size (compare Figs. 16 and 17 with Fig. 18). This stage, represented by Fig. 18, really marks the beginning of the growth period and from now on the increase in the size of the cells is more evident. When Fig. 18 is examined it can be seen that no continuous spireme is formed. The leptotene threads remain separate and may be distinguished from each other. Owing to the great number of these threads it was impossible to count them.

The primary spermatocytes now undergo synizesis. The leptotene threads migrate to one pole of the nucleus (Fig. 19), the so-called "synaptic pole" and arrange themselves in parallel pairs. This paired arrangement is very distinct and has been observed in a great many cells. While this is going on, two large round bodies, of more or less equal size, and surrounded by clear spaces (Fig. 19, *k*) make their appearance in the cytoplasm. These bodies were first observed in a smear preparation which was heavily stained. They stained exactly like the chromatin. In preparations which were strongly destained no trace of them was found. However, when these latter preparations were restained, the bodies loomed up with exceptional clearness. It is thus seen that although these structures stain like chromatin, yet they differ from chromatin in their affinity for nuclear dyes, and, in all probability, they are chemically different from chromatin. Wilson ('13), first described a similar body in *Pentatoma*, and called it a "chromatoid body." In *Cambarus virilis*, I ('14) have found a pair of chromatoid bodies appearing at about the same stage in the developing spermatocyte as in *Cancer magister*. After the leptotene stage, the chromatoid bodies of *Cancer magister* persist within the cells, and their subsequent history will be outlined when the respective stages of the maturation are dealt with.

Synapsis soon sets in. The parallel threads become more closely paired at the synaptic pole and the cell enters the pachytene stage, in which the pairs of leptotene threads fuse into thick gemini (Fig. 20). Here the union appears complete as no traces could be found of the longitudinal furrows which separated the original pairs of parallel threads.

After remaining fused for some time, the components of each geminus begin to unravel. Along every geminus a longitudinal split makes its appearance (Fig. 21), and simultaneously with this there is an opening of the paired threads at one end, while remaining attached at the other end, thereby producing figures which appear like 8, V, or less commonly like U (Figs. 21 and 22).

Another longitudinal split soon occurs along the arms of each geminus. In Fig. 22 the beginnings of this second longitudinal furrow are clearly visible in the two arms of the 8 which is located in about the center of the nucleus. By means of these two longitudinal cleavage planes, four chromatin threads are formed, distinctly opened up at one end and temporarily fused at the opposite end. The two pairs of these threads continue to diverge in opposite directions until they ultimately form X's (Fig. 23).

The pairs of threads on opposite sides of the central fusion point of each X now migrate closer to each other until they come to lie almost parallel. The central fusion point next disappears, and four thin threads arranged in two parallel rows are produced. A hasty glance at this arrangement makes it appear as if the geminus from which the four threads were derived, was traversed by a longitudnal and a transverse cleavage plane (see Fig. 23). However, prolonged and careful study reveals the true nature of the case, that the threads under discussion are the results of two longitudnal splits of each geminus.

The tetrads are soon formed. Each thin thread shortens and thickens into a spherical chromatin mass (Figs. 23 and 24), so that very soon every geminus is changed into four spherical chromosomes, representing a tetrad (Figs. 23–25). Each tetrad thus contains four univalent chromosomes. In the next step pairs of these univalent chromosomes fuse, resulting in two large bivalents attached to each other by linin strands. This condition is particularly well shown in Figs. 24 and 25. The condensation of the bivalents continues until they are changed into dumb-bell shaped structures (Fig. 25). The growth process is now completed and the cell is ready to undergo reduction.

It is quite evident from the above discussion that in *Cancer magister* we apparently have to deal with a case of parasynapsis, or side by side conjugation of the chromosomes. This conclusion was reached after prolonged observations upon numerous excellent smear preparations, which contained an abundance of synapsis material. The essential steps closely resemble those of *Cambarus virilis* (Fasten, '14).

(*b*) *Reduction Division.*—In the last stages of the growth period, the nuclear wall begins to break down (Fig. 24). The two centrosomes, which have been formed by a division of the original centrosome, migrate to opposite poles, and thin spindle fibers make their appearance between them and the chromosomes. The chromosomes are soon pulled to the equator of the cell to undergo reduction. In Fig. 25, the dumb-bell shaped bivalents are seen arranging themselves. Here the chromatoid bodies can also be clearly discerned at opposite poles. Each of these bodies is surrounded by its characteristic clear space and may be easily recognized from the ordinary chromosomes.

In the metaphase period (Fig. 26), all of the chromosomes are grouped in the equatorial plane and appear as large dumb-bells. When the component bivalents of each dumb-bell are examined, they are found to be large spherical bodies, about twice the size of the spermatogonial chromosomes. Furthermore, these bivalents do not show the equational furrows where the equational division of the second spermatocyte will take place. Binford ('13), in describing this stage in *Menippe mercenaria* claims that the chromosomes in the equatorial plate of one of his preparations were found to be ring-like in appearance. In other preparations which were destained to the degree of removing all the stain from the cytoplasm and the achromatic figure of the metaphase, this same investigator asserts that he could distinguish the individual four chromosomes of each tetrad. In *Cancer magister* no such results were obtained in spite of the fact that a great many smears and sections were carefully examined.

In polar views of the metaphase stage sixty chromosomes have been distinguished (Figs. 27 and 28). These are generally oval in shape; some of them being larger than others and they are distributed throughout the equatorial plane. Figures 27 and 28 are drawings of polar views as seen in smear preparations. Similar counts of polar views in sectioned material have corroborated this number for the reduction division.

The chromatoid bodies always migrate undivided to opposite poles of the cell. In many cases they occupy positions along the spindle fibers (Figs. 26, 29 and 30), while in other cases they may be seen in the cytoplasm (Figs. 31 and 32). When they occupy positions along the spindle fibers, one would be easily misled into regarding them as accessory chromosomes, especially so if no attention were paid to the various stages of the growth period. Wilson ('13) has cited numerous cases in which investigators have undoubtedly confused chromatoid-like bodies with accessory chromosomes. After citing these instances, Wilson, on p. 403, then makes the following significant remarks:

"Such facts make it clear that the presence of sex-chromosomes can not safely be inferred alone from the presence of chromosome-like bodies lagging on the spermatocyte-spindles, or lying near one pole. The presence of compact, deeply staining nucleoli during the growth-period is by itself equally indecisive. In some cases the 'plasmosome,' especially after certain fixatives such as Bouin's fluid, may stain quite as intensely as the chromosome-nucleoli with haematoxylin, safranin and other dyes (cf. Gutherz, '12). Decisive evidence regarding these bodies can only be obtained by tracing their individual history and by accurate correlation of the chromosome-numbers in the spermatogonial and spermatocyte-divisions. It hardly need be added that great caution is necessary in dealing with difficult material in which for any reason such a test cannot be completely carried out."

The anaphase stage (Fig. 29) follows upon the metaphase. The bivalents of the dumb-bells are separated and pulled to opposite poles. The chromatoid bodies also migrate in these directions. The division process continues and gradually the primary spermatocyte enters the telophase stage (Figs. 30 and 31). In the final telophase (Fig. 32), the chromosomes which have completely migrated to opposite poles become surrounded by thin nuclear walls. The cytoplasm has constricted off into two distinct portions and during this process the spindle fibers have also been constricted so that at their center a thickened "zwischenkörper" is formed. When this stage is completed two secondary spermatocytes are produced, each possessing one of the chromatoid bodies within the cytoplasm (Fig. 32).

C. *Secondary Spermatocyte Stage.*

The secondary spermatocytes formed during the ultimate telophase stage of the reduction division, undergo immediate transformations. No rest period could be determined. This is similar to what occurs in *Astacus fluviatilis* (Prowazek, '02), in *Menippe mercenaria* (Binford, '13), and in *Cambarus virilis* (Fasten, '14). The cells assume the metaphase stage (Fig. 33), and the chromosomes line up in the equator of the spindles in the form of dumb-bells. When polar views of these chromosomes are studied in preparations which have been greatly destained, sixty of them may be counted (Figs. 34 and 35). These chromosomes are about half the size of those found in the reduction division (compare Figs. 34 and 35 with Figs. 27 and 28). As for the chromatoid body, it generally lies at or near one pole of the cell, leaving the other pole without any such element.

The anaphase (Fig. 37) and telophase (Figs. 38 and 39) stages follow each other in logical sequence, resulting in the division of the secondary spermatocytes to form spermatids. Two types of spermatids (Fig. 40), are thus formed in equal numbers; one type which contains a single chromatoid body in the cytoplasm (Figs. 40 and 41), whereas, the second type is without this body (Figs. 40 and 43).

D. *Transformations of the Spermatids into Spermatozoa.*

The spermatids produced are, at first, small and their nuclei contain large masses of chromatin material which stain intensely with nuclear dyes (Fig. 40). The cytoplasm is homogeneous throughout and within it a rather prominent centrosome is found. In the second type of spermatid developed, the cytoplasm, in addition to containing the centrosome, also possesses the chromatoid body (Figs. 40 and 41).

The first noticeable changes undergone by the spermatids in transforming into spermatozoa, occur in the nucleus. The chromatin mass of the nucleus is gradually reduced to such a degree that it loses its intense staining qualities, and becomes quite homogeneous in consistency (Figs. 40–47). At first the large chromatin clumps break up into granular masses (Figs. 40, 43 and 44). Then these fragment still more completely until in the final stages there remain, respectively, three round chromatin bodies (Fig. 45); then two (Figs. 42 and 46), and ultimately one (Fig. 47). This remaining chromatin structure is spherical, stains intensely black with Heidenhain's haematoxylin and occupies the center of the nucleus. It may be said to be a nucleolus-like body which resembles a karyosome.

Both classes of spermatids produced undergo similar changes of the nucleus. In the second type of spermatid, however, an interesting change goes on in the cytoplasm, simultaneously with the transformations of the nucleus. Here the chromatoid body wanders from its position within the cytoplasm to the periphery of the cell and is soon expelled to the outside (Figs. 40–42), thus playing no further rôle in the transformations. From now on all the spermatids are exactly alike and they undergo the same modifications.

At about this time a densely staining mass makes its appearance in the cytoplasm (Figs. 46 and 47, *m*). This mass has been called a mitochondrial mass by Koltzoff ('06) and Binford ('13). It stains like the chromatin of the nucleus, and first makes its appearance at about the stage in the spermatid transformations where the chromatin of the nucleus becomes reduced to two clumps (Fig. 46). As to whether this mass consists of mitochondria or not is a debated question. The fact of the matter is that cytologists themselves are not clear as to which bodies within the cell are mitochondria and which are not (see Cowdry, '16). In the cells under consideration, no traces of mitochondria have been observed in the earlier stages of the maturation. This darkly-staining mass makes its appearance only after most of the chromatin within the spermatid nucleus has been much reduced. This would lead one to suspect that the mass might consist of chromatin which has diffused out of the nucleus and has accumulated within the cytoplasm (see Figs. 43–47). At any rate, this seems a likely probability.

The spermatids now reveal the following distinct structures, (1) a rather homogeneous nucleus (Fig. 47, *n*), with a karyosome-like body in the center, (2) a cytoplasm in which are found (3) a centrosome (Fig. 47, *c*), and (4) a mitochondria-like mass (Fig. 47, *m*), which stains like chromatin. These elements must be clearly kept in mind in order to follow up the later changes of the spermatids. In linking up these changes it is also necessary to exercise great care. Sectioned material, while helpful, is by itself wholly inadequate for this purpose. Smear preparations of the entire cells, on the other hand, give a true picture of what happens, and these were used almost entirely in the study of transformations.

The nucleus wanders to one pole of the spermatid (Figs. 48–50, *n*), while at the opposite pole a clear vacuole (Fig. 50, *v*), makes its appearance. Sometimes two clear openings (Figs. 48–49, *v*) may be seen, but these later flow together into a single one (Fig. 50, *v*). At the same time the mitochondrial mass wanders in between the nucleus and the vacuole (Figs. 48–52, *m*), and ultimately fills this entire space (Fig. 53). The centrosome increases somewhat in size and takes a position in the center of the mitochondrial mass (Figs. 50–53, *c*).

The mitochondrial mass now transforms into a ring resembling a doughnut, and the centrosome comes to occupy the center of its inner open space (Figs. 53 and 54, *c*). The upper portion of the nucleus also becomes located in this space (Figs. 54–58, *n*). At the same time, the karyosome-like body, situated within the center of the nucleus (Figs. 47–53) migrates upward to the middle of the upper portion of the nucleus (Fig. 54) until it comes to lie directly below the centrosome.

Binford ('13) in describing the transformation of the spermatids of *Menippe mercenaria* claims, on p. 156, that "after the mitochondrial ring is completed the nucleus becomes widely separated from it and the capsule (Figs. 50 to 52). This, however, is not always the case. In two preparations from which Figs. 33 to 35 and 37 to 43 were drawn, the nucleus remained fitted closely on the capsule as shown in Fig. 43. As the two different conditions were obtained with the same fixing fluid it is hardly probable that the difference was caused by the fixing."—In sections of testicular material of *Cancer magister*, a few of the transforming spermatids showed the conditions which Binford describes, but in smear preparations not a single such instance was discernible. I therefore suspect that Binford had to deal with defects which are often produced in cytological material which is prepared by the fixation and the sectioning methods.

Simultaneously with the last-mentioned changes, a second vacuole (Fig. 54, *v′*), makes its appearance in the anterior extremity of the original first (Fig. 54, *v*), or primary vacuole. At first this second vacuole looks like a small bubble of liquid which stains rather darkly with Heidenhain's haematoxylin. It soon increases in size, becomes more distinct (Figs. 54–57, *v′*), and stains somewhat lighter. It is quite evident that Binford's ('13) so-called "inner tubule" formed during the spermatid transformations of *Menippe mercenaria*, is the same structure which I have called the second vacuole in *Cancer magister*. My preparations, however, show no such stages which Binford describes and pictures (Figs. 52–60 of Binford, '13), for the development of this structure.

During this time the centrosome and the karyosome-like body of the nucleus unite (Figs. 54–56), and elongate into a rod-like structure (Figs. 57 and 58), the so-called central body (Figs. 56 and 57, *b*). At first the central body looks like a dumb-bell (Figs. 56 and 57, *b*), but as it lengthens out, it loses this appearance, becomes more rod-like (Figs. 58–60), and at the same time penetrates the inner or proximal portion of the second vacuole. While all these changes are going on the primary and secondary vacuoles are gradually transforming into primary and secondary vesicles (see Figs. 54–60, *v* and *v'*), and from now on they will be designated by the latter names.

At about the stage represented by Fig. 59, an opening makes its appearance in the middle of the outer, or distal end of the second vesicle. Simultaneously with this, the central body elongates still more (Fig. 59) and its outer extremity seems to hollow out into a thin tube (Figs. 60–62) which soon connects up with the distal opening in the secondary vesicle. As the outer end of the central body hollows out, a ring of densely staining material makes its appearance around the outer opening of the second vesicle (Figs. 61–63, *d*). In Heidenhain's haematoxylin this ring stains intensely black like the centrosome or the chromatin. This ring may be spoken of as a chromatin-ring and it becomes more distinct as the distal end of the central body hollows out (Figs. 61–64, *d*). Going hand in hand with these modifications are those which take place in the mitochondria-like ring and the nucleus. These two elements fuse into a single structure, a sort of nuclear-mitochondrial cup (Fig. 62, *h*), which stains quite homogeneously, although some part of the mitochondrial mass seems to persist around the wall of the first vesicle and stains more intensely than the rest of the cup. (see Fig. 62, *h*).

As the transformations continue, the second vesicle fits more compactly into the first vesicle (Figs. 63 and 64). The outer portion of the central body has completely changed into a thinwalled hollow tube, which stains lightly. The inner portion of the central body is solid, stains intensely with nuclear dyes and is embedded in the center of the nuclear-mitochondrial cup, immediately below the wall of the first vesicle. Figs. 63–65 show these structures nicely. Now the radial arms or rays of the spermatozoön (Figs. 63 and 64, *r*) make their appearance. They originate as outgrowths from the nuclear-mitochondrial cup, and in the finished state they are stout structures with pointed extremities (Figs. 63 and 64, *r*).

Soon the spermatids are completely transformed into mature spermatozoa, and in this state (Figs. 65 and 66) they look like ovoid, or spheroid structures, in which the radial rays are tightly coiled around the nuclear-mitochondrial cup. Figs. 65 and 66 are drawings of mature spermatozoa as viewed, respectively, from the side and bottom. An examination of these figures shows the central body (*b*) located in the middle, and surrounding it in order of sequence are the second vesicle (*v′*), the first vesicle (*v*) and the nuclear-mitochondrial cup (*h*). In Fig. 65, the structure of the central body (*b*) consisting of a hollow distal end and a solid proximal end, can be distinctly observed. In this figure may be also seen the densely staining chromatin-ring (*d*), located at the upper end of the secondary vesicle.

When the mature spermatozoa are studied in smear preparations which have been fixed with Bouin's fluid and stained by the iron-haematoxylin and acid-fuschin methods, then the second vesicle stains a dark amber color, while the primary vesicle takes almost no stain and remains clear. In sections fixed with Flemming's fluid and stained similarly, the second vesicle remains more or less transparent, while the first vesicle stains a dark brown.

The mature spermatozoa are at first free, but when they make their way into the vasa deferentia they are surrounded by the membranous pouches or spermatophores already mentioned under the discussion of the testis. Fig. 67 shows one of these spermatophores when it is first removed from the vas deferens of the living crab. Notice the numerous spermatozoa which are compactly stored within its interior.

5. Opening of the Mature Spermatozoa.

The mature spermatozoa within the spermatophores are dormant structures, but when they are removed from the spermatophores and placed in fluids whose concentration is less than sea water, they undergo interesting changes. In many ways these are similar to the changes which Binford ('13) describes for the spermatozoa of *Menippe mercenaria*.

The method employed in studying the opening of the mature spermatozoa of *Cancer magister* was similar to that employed by Binford ('13). Numerous spermatophores suspended in either crab's body fluids, or sea water, were placed on a slide and covered with a cover-glass. By applying pressure to the cover-glass many of the spermatophores were ruptured, thus liberating the spermatozoa. These could then be examined under the high powers of the microscope. By allowing numerous salt solutions, already mentioned under the section on 'Materials and Methods,' to diffuse under the cover-glass, all of the changes in the opening up process or the so-called explosion of the spermatozoa could be followed out quite accurately. Many of these spermatozoa in various stages of their explosion were fixed and stained on the slides and were then used for later study and comparison.

In the crab's fluids, in sea water, or in solutions of NaCl, KCl, $CaCl_2$, $NaNO_3$ and KNO_3 which are isotonic with sea water, almost no change occurs. Usually the rays of the nuclear-mitochondrial cup unravel, revealing spermatozoa which contain either three (Fig. 68) or four (Fig. 69) rays. In hypotonic solutions of the last-mentioned salts, the spermatozoa undergo an explosion, and change considerably in appearance. Osmotic pressure, undoubtedly, accounts for this explosion as was suggested by Koltzoff ('06).

The first step in the explosion of the spermatozoa is the extrusion of the second vesicle. This vesicle normally surrounds the central body and is embedded in the first vesicle (Figs. 70 and 71). When the second vesicle begins to extrude, it swells somewhat (Figs. 72–76, *v′*) in size and at the same time it stretches the upper portion of the first vesicle and makes it appear like a thickened ring (Figs. 72–76, *s*). Simultaneously with this, the hollow distal end of the central body which is also extruded, exerts a pull on its solid proximal portion, transforming it into a spine-like structure (Figs. 72–76, *b*), which stains intensely black with Heidenhain's hæmatoxylin. While these changes are going on, the nuclear-mitochondrial cup loses its rays and rounds out into a spherical body.

When the second vesicle has been completely extruded (Fig. 75, *v′*), then the first vesicle (Fig. 75, *v*) commences to evert and continues this process until it is completely turned inside out. These steps may be observed in Figs. 75–79, *v*. During the eversion, the darkly staining proximal end of the central body forces upward on its tubular distal portion until the latter is finally extruded completely to the outside (see Figs. 75–77, *b*).

The completely exploded spermatozoa present the appearances represented in Figs. 78 and 79. The upper portion consists of the second vesicle (*v′*), in the interior of which is contained the everted first vesicle (*v*), with its upwardly projecting spine-like body (*b*). The lower portion consists of the nucleo-mitochondrial cup (*h*), which has transformed into a more or less spherical structure. In many cases, stages like those shown in Fig. 80 were seen. These evidently are exploded spermatozoa in which the second vesicle has completely ruptured and disintegrated. Binford ('13) has observed similar conditions in *Menippe mercenaria.*

6. Discussion.

A. *Synapsis.*

During the last few years the parasynaptic view of chromosomal conjugation has been established in numerous species of animals. In 1900, Von Winniwarter first advocated parasynapsis amongst the mammals, but the view did not become firmly established until the Schreiners ('04, '05, '06, '07 and '08) published their important researches on the germ cells of many animals including mammals, birds, reptiles, amphibians, fish, molluscs and annelids. Of late the parsaynapsis view of Von Winniwarter and the Schreiners has been extended to a great many additional forms, and excellent reviews of the vast literature on this subject may be found in the recent publications of Montgomery ('11), Wilson ('12), Fasten ('14) and Wenrich ('16).

Among the Crustacea, parasynapsis has been established in some of the Copepoda (Lerat, '05; Matschek, '09; McClendon, '10; and Kornhouser, '15), and also in one of the Decapoda (Fasten, '14). In the decapod crustacean *Cambarus virilis*, I ('14) showed that during the growth period, the chromosomes conjugate in parasynaptic fashion, and as already pointed out elsewhere in this paper, this is the type of conjugation which occurs in *Cancer magister*. In both *Cancer magister* and *Cambaras virilis*, the great difficulty encountered in the study of synapsis was the immense number of chromosomes. However, after prolonged and careful study of the various stages in the growth period of these animals, one finds it rather difficult to interpret the conjugation of the chromosomes in any other way than by parasynapsis.

B. *The Chromatoid Bodies.*

Ever since Wilson ('13) called attention to a chromatoid body in the spermatogenesis of *Pentatoma*, similar structures have been described during the spermatogenesis stages of other forms. In *Cambarus virilis* (Fasten, '14), two such bodies were found which could be traced into the spermatid stages and then all traces of them were lost. In *Cancer magister*, a pair of chromatoid bodies make their appearance during the synizesis stage of the growth period, and during the reduction division these pass to opposite poles, so that the secondary spermatocytes each possess a chromatoid body. During the equation division, this body passes undivided to one pole, resulting in two types of spermatids, one type possessing a chromatoid body, while the other type is minus such a structure. It is also of interest that the chromatoid body is eventually expelled from the first type of spermatid thus playing no further part in spermatogenesis.

Concerning the nature and function of the chromatoid body, very little can be said. In only two forms, namely *Pentatoma*, (Wilson, '13) and in the decapod under consideration *Cancer magister*, has the full history of this structure been traced, and in both cases, it is expelled from the spermatids, thus appearing to play no definite rôle in the mature spermatozoa. Wilson ('13), in discussing the chromatoid body, makes the following trite remarks on page 402: "The nature of the chromatoid body thus remains problematical, but the facts are worthy of serious attention for another reason. Were the chromosomes very small, numerous, closely crowded, or otherwise unfavorable for exact study, and could not the entire history of the chromatoid body be so clearly traced, even an experienced observer might fall into the most confusing error concerning the relations of the chromosomes."

7. SUMMARY.

1.. During the latter part of June and the early part of July the testicular lobes of *Cancer magister* are in the best shape for the study of spermatogenesis.

2. Two spermatogonial divisions can be recognized, and these ultimately form the resting primary spermatocytes.

3. Sometimes, larger and more intensely staining cells are found interspersed among the spermatogonia. These are the nutritive cells, and it seems very probable that they have originated from a transformation of some of the spermatogonia. The nuclei of the nutritive cells are irregular in shape and many of them possess amœboid processes. In sections of some of the nutritive cells two or more nuclei are oftentimes found, and this might easily mislead one into concluding that amitosis occurs amongst them.

4. The resting primary spermatocyte undergoes a growth period, during which thin leptotene threads are produced through the fragmentation of the chromatin. No continuous spireme is formed as the leptotene threads appear distinct and separate.

5. During the growth period pairs of leptotene threads migrate to the synaptic pole of the cell, become arranged in parallel fashion and soon fuse parasynaptically.

6. During the synizesis stage of the growth period a pair of densely staining chromatoid bodies make their appearance in the cytoplasm. These are surrounded by clear areas and may have originated from some of the chromatoid masses found within the cytoplasm of some of the earlier stages in the spermatogenesis.

7. The first spermatocyte division is reductional. In the metaphase stage the chromosomes line up as dumb-bells, which are composed of pairs of bivalents. The chromatoid bodies pass undivided to opposite poles of the cell.

8. A polar view of the metaphase stage of the reduction division reveals sixty chromosomes distributed throughout the entire plane of the equator.

9. The division of the primary spermatocytes results in secondary spermatocytes, each of which contains a chromatoid body.

10. The second spermatocyte division is equational and immediately follows the reduction division. A polar view of the metaphase stage of the equational division reveals sixty chromosomes which are about half the size of those found during the reduction division.

11. The chromatoid body passes undivided to one pole during the division of the secondary spermatocyte, resulting in the formation of two classes of spermatids, one of which contains the chromatoid body, while the other is without such a structure.

12. The chromatoid body is soon expelled from the spermatids which contain it, thus making all the spermatids alike in structure and appearance.

13. The nucleus of the spermatid loses its large quantity of intensely staining chromatin, while at the same time a mitochondria-like mass makes its appearance in the cytoplasm. Also one or two vacuoles are formed in the cytoplasm.

14. As the transformations go on the nucleus becomes elliptical and wanders to one pole of the cell. The vacuoles fuse into a single large vacuole which then takes a position at the opposite pole of the cell. The mitochondria-like mass wanders in between these two structures, becomes ring-like, and within its center and above the karyosome-like body of the nucleus, the centrosome becomes stationed. Soon a second vacuole makes its appearance at the distal end of the first one.

15. The two vacuoles gradually transform into the first and second vesicles. The centrosome and karyosome-like body of the nucleus become fused into the central body, which runs through the middle of the second vesicle, while the nucleus and mitochondria-like ring unite into a nucleus-mitochondrial cup from which the rays of the spermatozoön are produced.

16. The mature spermatozoa are oval bodies tightly packed within membranous spermatophores.

17. When the mature spermatozoa are surrounded with salt

solutions possessing a lower osmotic pressure than either the crab's body fluids or sea water, they undergo an interesting explosion in which the vesicles and the central body are completely everted, while at the same time the nuclear-mitochondrial cup rounds out into a spherical structure.

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9. DESCRIPTION OF PLATES.

All the illustrations in the accompanying plates were made with the aid of the camera lucida. Figs. 1–66 and 70–80 were drawn under Zeiss apochromatic objective 2 mm. Hom. Im., Comps. ocular 12,[1] at a magnification of 2,720 times. Figs. 67–69 were drawn under achromatic lenses at an approximate magnification of 1,400 times.

ABBREVIATIONS.

b = central body.
c = centrosome.
d = chromatin ring.
h = nuclear-mitochondrial cup.
k = chromatoid bodies.
m = mitochondria-like mass.
n = nucleus.
r = rays or arms of spermatozoa.
v = first or primary vacuole (first or primary vesicle).
v' = second or secondary vacuole (second or secondary vesicle).

EXPLANATION OF PLATE I.

FIG. 1. Resting primary spermatogonial stage.

FIG. 2. Spermatogonial prophase, showing fragmentation of chromatin. Within the cytoplasm two large, heavily staining masses, probably chromatoid masses can be distinguished.

FIG. 3. Late spermatogonial prophase. Note the compact chromatin clumps within the nucleus.

FIG. 4. Metaphase, primary spermatogonium.

FIG. 5. Anaphase, primary spermatogonium.

FIGS. 6 and 7. Telophase, primary spermatogonium.

FIG. 8. Resting secondary spermatogonial stage.

FIGS. 9 to 12. Nutritive cells. Observe the irregular nuclei and the fatty globules within the cytoplasm.

FIG. 13. Nutritive cell, in which the nucleus shows a constriction in the middle which appears to be suggestive of amitosis.

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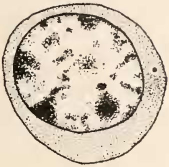
[1] I wish to express my indebtedness and gratitude to the Department of Zoölogy of the University of Wisconsin for the loan of a set of Zeiss apochromatic lenses (2 mm. oil immersion objective and compensating oculars 4, 8 and 12), which were of great service in the problem under consideration.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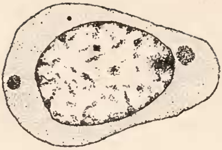
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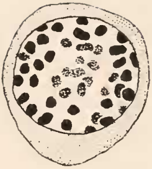
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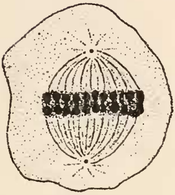
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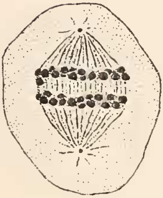
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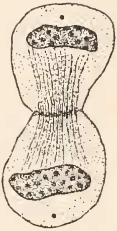
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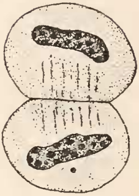
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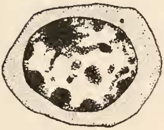
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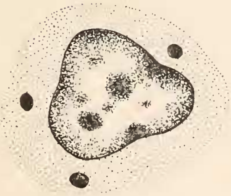
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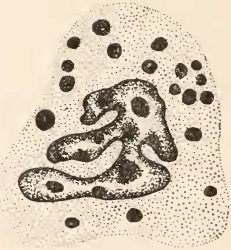
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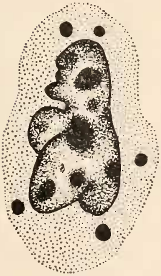
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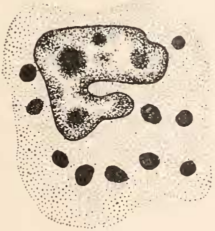
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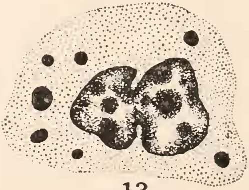
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EXPLANATION OF PLATE 2.

FIGS. 14 and 15. Nutritive cells, with two nuclei, suggestive of amitosis.

FIGS. 16 and 17. Early prophases, primary spermatocyte stage. In Fig. 17 two darkly staining chromatoid masses can be seen within the cytoplasm.

FIG. 18. Leptotene stage.

FIG. 19. Synizesis and synapsis stage. In this stage the leptotene threads have a paired parallel arrangement at the synaptic pole of the cell. The chromatoid bodies (*k*), and the centrosome (*c*), are also visible in the cytoplasm.

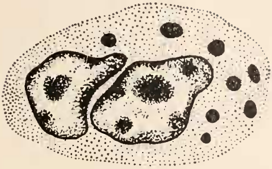
FIG. 20. Pachytene stage. The paired leptotene threads have united into thick gemini.

FIGS. 21 and 22. Diplotene stage.

FIG. 23. Postdiplotene stage.

FIG. 24. Tetrad formation. In this stage the nuclear wall begins to break down.

FIG. 25. Tetrads transformed into dumb-bells. The cell is entering the metaphase and the chromatoid bodies, surrounded by clear spaces are seen at opposite poles.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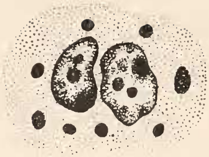
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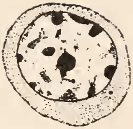
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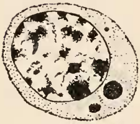
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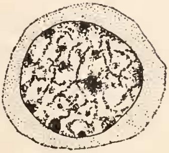
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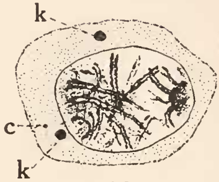

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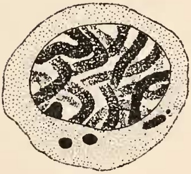
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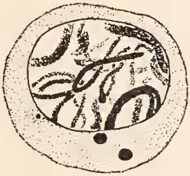
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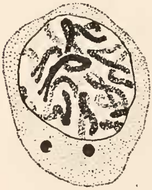
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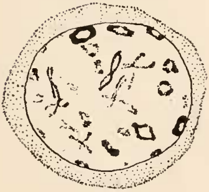
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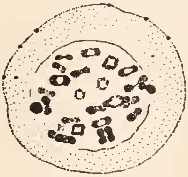
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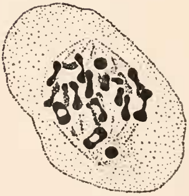
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Explanation of Plate 3.

Fig. 26. Metaphase, primary spermatocyte showing chromatoid bodies along the spindle fibers at opposite poles.

Figs. 27 and 28. Polar views, primary spermatocytes, showing 60 chromosomes.

Figs. 29 to 32. Anaphase and telophase stages of primary spermatocytes showing the chromatoid bodies at opposite poles. In Fig. 32 the chromatoid bodies are observed to remain in the cytoplasm.

Fig. 33. Metaphase, secondary spermatocyte, showing a single chromatoid body at one pole.

Figs. 34 and 35. Polar views, secondary spermatocytes, showing 60 chromosomes.

Figs. 36 and 37. Anaphase stages, secondary spermatocytes, showing different positions which the chromatoid body may occupy in the dividing cell.

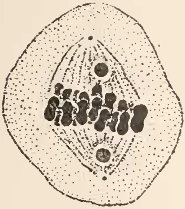
Figs. 38 and 39. Telophase stages, secondary spermatocytes. The single chromatoid body is at one pole.

Fig. 40. The two types of spermatids formed, one without the chromatoid body, and the other containing it. The centrosomes are the minute dark granules found in the cytoplasm of both these types of spermatids.

Fig. 41. Early stages in the transforming spermatid which contains the chromatoid body.

Fig. 42. Stage in which the chromatoid body is expelled from the spermatid that contained it.

Figs. 43 to 47. Early transformation stages of spermatids which are minus the chromatid body. Note the reduction of the chromatin and the appearance of the mitochrondria-like mass (*m*) in the cytoplasm. In Fig. 47 the single karyosome-like body occupying the center of the nucleus may be seen.



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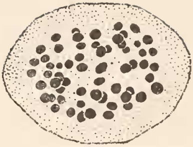

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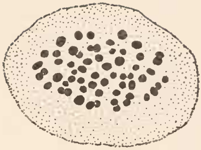

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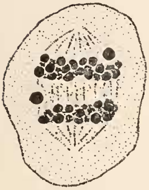

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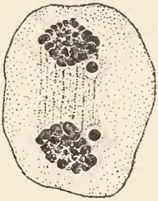

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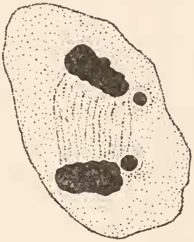

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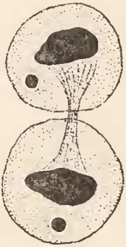

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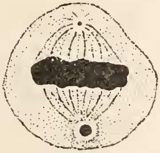

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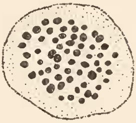

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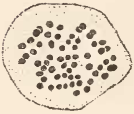

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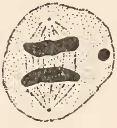

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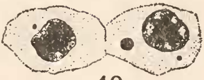

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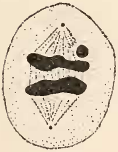

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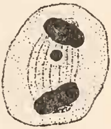

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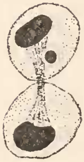

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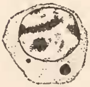

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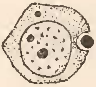

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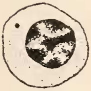

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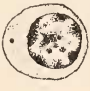

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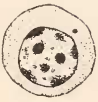

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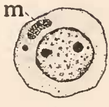

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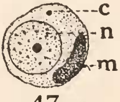

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N. FASTEN, DEL.

EXPLANATION OF PLATE 4.

FIGS. 48 to 53. Successive stages in the transformation of the spermatid, resulting in the primary vacuole (*v*) being formed at one pole, while the nucleus (*n*) occupies the opposite pole, and in between them the mitochrondria-like mass (*m*) and the centrosome (*c*) take their position.

FIG. 54. The secondary vacuole (*v′*) makes its appearance at the distal end of the first vacuole (*v*).

FIGS. 55 to 58. Stages in the transformation of the spermatid in which the primary and secondary vacuoles transform into distinct vesicles, and the central body (*b*) assumes a dumb-bell appearance.

FIGS. 59 to 62. Spermatid transformations showing the hollowing out of the distal end of the central body; the formation of the chromatin-ring (*d*), and the fusion of nucleus and mitochondria-like mass into a nuclear-mitochondrial cup (*h*).

FIGS. 63 and 64. Formation of the rays (*r*) of the spermatozoön.

FIGS. 65 and 66. Side and bottom view of mature spermatozoa, showing details of structure.

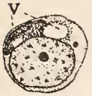
FIG. 67. Spermatophore, filled with mature spermatozoa.

FIGS. 68 and 69. Three- and four-rayed types of spermatozoa, as seen suspended in the body fluid of *Cancer magister*.

FIGS. 70 to 78. Successive stages in the explosion of the mature spermatozoön when surrounded with salt solutions of lower osmotic density than sea water.

FIG. 79. Spermatozoön which has exploded in distilled water.

FIG. 80. Exploded spermatozöon in which the secondary vesicle has completely disintegrated.



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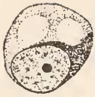

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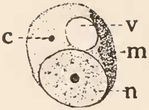

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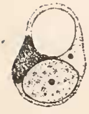

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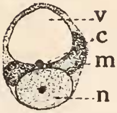

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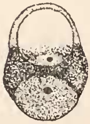

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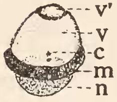

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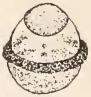

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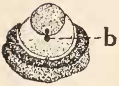

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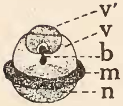

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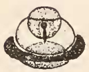

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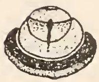

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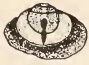

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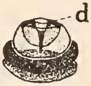

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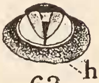

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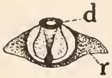

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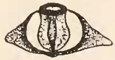

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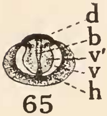

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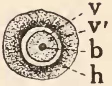

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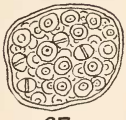

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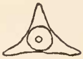

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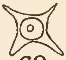

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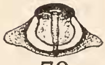

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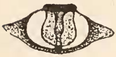

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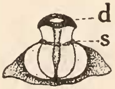

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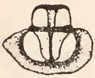

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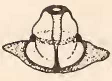

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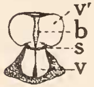

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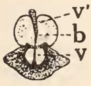

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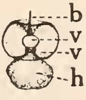

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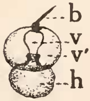

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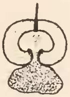

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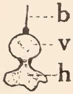

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N. FASTEN, DEL.

OBSERVATIONS ON THE STRUCTURE OF PROTOPLASM BY AID OF MICRODISSECTION.

WILLIAM SEIFRIZ.

TECHNIQUE.

The introduction of the Barber pipette holder brought into use an exceedingly ingenous technique which promises to open up a great field of research, in which not only many of the results got from fixed material can be confirmed or refuted, but in which observations on the structure and behavior of living protoplasm can be made with an accuracy and certainty not otherwise possible. This instrument, originally designed to hold miniature pipettes for isolation and injection work, is equally well adapted for the manipulation of glass dissection needles. Each needle is held in a three-movement clamp and extends into a small moist chamber placed on the stage of the microscope. The vertical needle-tips project up into a hanging drop in which the material to be dissected is suspended.

A description of this instrument and of the technique connected with it was first published by Barber (1914). Chambers (1915) has given a brief account of its use, and later (1917*c*) published a full description of the instrument and of the ways of making micro-needles.[1]

LITERATURE.

The literature published on microdissection studies is limited practically to the articles of G. L. Kite and Robert Chambers. Both of these men employed a Barber instrument essentially identical with that used by the writer. A list of their publications is appended to this paper.

TERMINOLOGY.

A serious difficulty encountered by the investigator in this field is the limited vocabulary which is at his disposal for the definition and description of the phenomena observed. We are little better off in choice of words than were the early microscopists whose knowledge of protoplasm was fully indicated by such adjectives as viscous, elastic, hyaline, etc.

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[1] It is of great importance in making sharp needles to have a minute flame. A very satisfactory micro-burner can be made—as was suggested to the writer by Dr. E. E. Free—from the smallest size (no. 27) hypodermic injection needle by snipping off the bevelled end with sharp shears.

In the possible range in consistency between the two extreme conditions of a colloid, *i. e.*, extreme liquidity and the solid state, the writer will distinguish ten degrees of viscosity, namely—watery, very liquid, liquid, slightly viscous, rather viscous, decidedly viscous, very viscous, extremely viscous, gel, and rigid gel—and has employed these expressions, for want of better ones, to describe the comparative viscosity of protoplasm. Their use will eliminate, in part, the vagueness from such general terms as liquid and viscous.

Protoplasm, which includes "all the living components of the cell-body" (Strasburger, 1891, p. 13), possesses many lifeless inclusions which materially affect its consistency, and they must be taken into consideration when the viscosity of protoplasm is discussed. As hyaloplasm is usually restricted to the hyaline border of non-granular plasma, the term matrix is used to indicate the translucent and more homogeneous fluid in which the various included granules are suspended.

Protoplasm, as such, frequently possesses a consistency different from that of its matrix. The latter may be watery yet the density of the protoplasm in toto markedly greater. It is, therefore, important to keep clear the distinction between the protoplasm as a whole and the matrix in which the various inclusions are imbedded.

MATERIAL.

The experimentation leading to the following results was carried on in the Harpswell Laboratory at South Harpswell, Maine. The writer wishes to thank the director, Dr. J. S. Kingsley, for the use of a room in the laboratory and for many other privileges enjoyed there.

The chief problem for the botanical microdissectionist is the obtaining of material delicate enough to permit dissection by fine glass needles. Even the wall of the frail alga *Spirogyra* can be entered only by the sharpest and most substantial needle that can be made. All in all, the most satisfactory objects for both zoölogical and botanical workers are the ova of marine organisms, although the myxomycetes are in some respects still better, for we have in them the largest masses of pure protoplasm known.

The observations here published were made upon myxomycetes, pollen-tubes, the oögonia, ova, and embryos of *Fucus*, and the ova of *Echinarachnius*.

OBSERVATIONS.

MYXOMYCETES.

Plasmodia of *Ceratiomyxa*, *Badhamia*, *Arcyria*, *Cribraria* and *Fuligo* were studied.

The protoplasmic density of myxomycetes is such that a needle traverses the plasma as through water, although it exhibits a slightly viscid property, for inclusions are pushed ahead of and to the side of a needle before they actually come in contact with it. However, if the point of a needle is broken off, making of it a minute pipette, cytoplasm and small inclusions rush into the opening with great rapidity and from quite a distance.

The vegetative plasmodium of a myxomycete is of very liquid consistency and remains so no matter how thin the film or filament of streaming plasma may be. If the plasmodium is not active a film or isolated globule of its protoplasm gels very rapidly. The consistency becomes so dense that a moving needle leaves a permanent furrow.

The old appellation "naked protoplasm," much used in reference to the slime-moulds, is in important respects a misleading one. The surface layer of a plasmodium is a definite morphological structure. The membrane is very extensile, slowly contractile, and surprisingly tenacious for so delicate a layer. This superficial layer can be isolated and held by one needle while stretched to several times its length by the other.

Within the plasmodium are definitely bounded smaller masses of protoplasm, which are apparently normal and in every respect identical with the surrounding plasma bulk. The origin of these smaller included protoplasmic masses was not observed, but their presence is common and they are readily distinguished from oil or other liquid inclusions. Several of these globules were isolated and dissected. Their limiting membrane is exceedingly sensitive, breaking before a perceptible indentation can be noted. Further dissection gave convincing evidence that this membrane is a morphologically differentiated layer. On breaking a globule some of the liquid protoplasm escaped. The pellicle of the ruptured mass stood out prominently and could be handled with a needle as one would handle a hair. It was of no appreciable thickness, yet quite rigid, though easily bent with no indication of being soft or glutinous. It had, therefore, during dissection, undergone a change—the normal, and extremely sensitive membrane had become a tough rigid gel. Such behavior well supports our interpretation of the plasmodium pellicle—not a surface-tension membrane nor a secreted wall, but a bounding layer of denser protoplasm. The plasmodial membrane is, then, a gel of appreciable thickness, tenacious, very extensile, contractile, and glutinous.

Next in importance to the possession of a definite morphological membrane is the capacity for forming one. The evidence which dissection work on myxomycetes presents on this phenomenon is, briefly, that the membrane is instantly and repeatedly reformed when ruptured by a needle, provided the protoplasm is normal. This is true whether the dissection be performed on a dry cover or in water; that is, the capacity is unchanged whether air or water is the surrounding medium. If gelation has set in the capacity for membrane formation is lost, although it may persist surprisingly long.

The living substance of slime-moulds is non-miscible in water.

POLLEN TUBES.

Of the many pollen grains experimented with those of the large blue flag (*Iris versicolor*) were the most satisfactory. The grains are large and germinate readily in almost any per cent. of sugar solution. The pollen of the beach pea (*Lathyrus maritimus*) is a fair substitute.

Repeated irritation of a pollen tube puts an end to protoplasmic streaming, although in some instances streaming may continue even after a tube has been punctured, and a large amount of its contents lost. Streaming, however, is not accelerated by irritation, nor is there any indication of a rush of protoplasm to wounded regions. The active protoplasm of a pollen tube is of very liquid consistency. On ejection it gels, though it does so slowly. Brownian movement sets in shortly after.

Ejected masses of protoplasm from pollen tubes develop membranes immediately on being freed. The capacity for membrane formation persists one to two minutes. Within three minutes the escaped protoplasm has become quite viscous. The membrane formed is surprisingly tough. Fragments of it can be dragged into the escaped plasma mass.[1]

Escaping protoplasm shows no sign of miscibility. Isolated groups of inclusions exhibiting Brownian movement, after ejection, were carefully observed and in every instance the cytoplasmic matrix was distinctly visible.

FUCUS.

The eggs of *Fucus* develop from divisions of the contents of oögonia which arise from single superficial cells of the wall of conceptacles that cover the fruiting branches. If these oögonia are teased out at a very early age they can be entered by a sharp needle. Very soon, however, the outer wall (exochiton) becomes too hard to be penetrated.

Oögonia.

In consistency the protoplasm of young uninucleate oögonia is very liquid. The wall is thick (2–4 microns), tough, and highly resilient. Slightly older oögonia, but still uninucleate, also possess very liquid protoplasm. Stages in development between young uninucleate oögonia and almost mature oögonia can not be observed, as the tough outer wall does not permit of dissection.

Immature Ova.

The contents of nearly mature oögonia can be squeezed out of the heavy exochiton by crushing the conceptacle with tweezers.

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[1] It was the intention of the writer to investigate the structure of the vacuole, of which so many are produced in the growing pollen tube. Freed vacuoles hold their shape even where exposed around the border of the plasma mass, apparently free of surrounding cytoplasm. When punctured the vacuoles collapse immediately. This behavior suggests that there is an enclosing membrane, and thus that the vacuole is apparently not really a vacuole but a sac. (De Vries, 1885, p. 467.)

Unripe eggs obtained in this manner are still held together by a jelly mass. The earliest stages so obtained were of oögonia in which division was complete but the eight eggs still of pentagonal outline in profile and closely appressed.

The protoplasm is of liquid consistency but shows marked signs of an increased viscosity over that of young oögonia. It tolerates a great amount of ill-treatment without showing any signs of injury. After half an hour of dissection there was no indication of gelation, the viscosity of the protoplasm remaining the same. Brownian movement, that unfailing criterion of degeneration, was not seen.

Slightly older oögonia in which the eggs, though still closely appressed, have rounded up somewhat, show further increase in protoplasmic consistency to the slightly viscous stage.

The separating membranes of these closely appressed masses of protoplasm are exceedingly delicate and of inappreciable thickness.

The capacity for membrane formation is complete. A remarkable property of the protoplasm of a young *Fucus* ovum is the rapidity with which it is enclosed by a wall after a needle has severed the egg. The very elastic glutinous membrane, ordinarily barely visible, is sufficiently pliable to be made of appreciable thickness when pressure is applied laterally with a tendency to compress.

The above described behavior of the ripening *Fucus* egg leaves no doubt that the plasma is a non-miscible fluid.

The stage of development of the eggs just described must be borne in mind. These ova, of slightly viscous consistency and enclosed by a delicate membrane, are nearing the completion of their development. They are still closely appressed within the oögonium, with several hours intervening before they would have been discharged as ripe eggs with gelatinous contents and a thick, hyaline wall.

The protoplasm of eggs of more advanced oögonia is of greater density, namely, rather viscous. It flows readily, but slowly.

The last stage in the development of the eggs before their discharge is especially noticeable because of an increase in thickness of wall. The enclosing layer is now one half of a micron thick. Notwithstanding the possession of a heavier wall these eggs can be dissected with as much ease, ending in the same results as those less mature ova having but a thin pellicle as covering. Droplets can be pinched off, the eggs torn from within outwards, or rapidly severed by a needle, without any indication of escaping protoplasm. The severed parts in every case instantly round up into droplets with walls apparently identical with the parent wall. This capacity to form a wall instantly at a ruptured point through conversion of the cytoplasm into a semi-rigid gel is not influenced by relatively great changes in the concentration of the surrounding medium, for it is as pronounced in very saline water as in normal sea-water.

There is no evidence of miscibility on the part of the protoplasm.

Fully Mature Ova.

The protoplasm of mature, normally discharged eggs is decidedly viscous, noticeably more so than that of well-developed unripe eggs. Thus has the highest degree of viscosity been reached in the development of the ovum, the transition taking place during the last periods of growth.

The wall likewise has undergone a marked change and become a hyaline, rigid gel, 0.8–1.2 microns thick, still very pliable and extensile, slightly contractile and exceedingly adhesive.

The wall of the mature normal *Fucus* ovum is capable of constant repair and this capacity often persists to the very last in a dying egg. Not until gelation of the protoplasm is well advanced does a rupture of the wall fail to be closed by a rapid conversion of the plasma matrix into a rigid gel. The capacity for wall formation is, then, one of the last essential properties of the living substance to be lost.

In all stages of its development before its normal discharge the egg shows no injury in consequence of dissection. The mature egg, on the contrary, is very sensitive to dissection, although its behavior is extremely variable. Every precaution was taken to prevent a misinterpretation of results due to observation of degenerated protoplasm. Such precaution was found to be more necessary with the ova of plants than with those of animals. To determine that the ripe eggs used for dissection were normal, part of each lot used was kept and tested for fertility.

The response to dissection of ripe unfertilized ova may vary from immediate disintegration to the toleration of two severances. Many eggs are very easily pinched in half by two needles rapidly approaching from opposite sides. The halves frequently round up into perfect spheres which can be again severed to form smaller protoplasmic droplets. While some eggs will show but slight if any increase in viscosity after many minutes of slow movement of the needles, others will suffer little or no dissection before gelating or completely disintegrating. Mere puncturing of the egg will often cause gelation. Thus does the ripe egg exhibit in one instance great sensitiveness and in another apparent indifference to stimulus.

This variability is carried even to parts of the same egg. One half of a severed ovum may gelate immediately without forming a wall over the torn surface, while the other half tolerates still another severance before gelating and thus losing the capacity for wall-formation. The presence of the nucleus in one half of the egg may be responsible for this difference in behavior (Townsend, 1897), although frequently both halves develop enclosing walls.

The protoplasm of the living ovum is at all times non-miscible in sea-water. This is to be expected if the capacity for wall-formation persists as long as the protoplasm is alive. In no instance of eggs examined in the various stages of ripening, while mature, and in the brief period following fertilization when the wall was still penetrable, was the plasma found to pour out and mix with the surrounding water.

The Unicellular Embryo.

Fertilization in *Fucus* is readily accomplished if active sperm are placed on a slide with mature ova.

Half an hour after application of the sperm the protoplasm of the fertilized ovum is found to be still quite viscous. A little later it becomes more liquid, for it readily oozes out of a puncture. Further development of the unicellular embryo shows a continued decrease in protoplasmic density.

The wall immediately after fertilization shows no increase in thickness and is still very pliable, extensile, and contractile. A little later it stiffens up until no longer elastic, though slightly resilient and pliable in so far that permanent indentations can be made. It can not now be severed by two approaching needles. In those few instances where the tough wall was punctured, and the protoplasm forced out, the ejected mass immediately developed a membrane. This plasma membrane is exceedingly thin, and quite elastic. It is interesting to note that the freed protoplasm here forms a delicate pellicle—not the tough wall of the one-celled embryo from which it came, nor the substantial though glutinous egg-wall which it would have developed in repairing a tear before fertilization.

At all times, from the moment of fertilization until dissection is no longer possible, the embryonic protoplasm is non-miscible. The ejected plasma shows no tendency to mix with the surrounding sea-water until after gelating, *i. e.*, after death.

The Multicellular Embryo.

As the two-celled embryo stage is neared the former egg wall has lost all of its former elasticity and glutinosity. It behaves under pressure like a ball of thin celluloid, resisting light pressure and returning to its former shape, unless forced beyond a certain point, when depressions made are retained. The two-celled embryo is punctured with great difficulty. The wall of the four-celled embryo is a very substantial affair, two microns in thickness, highly resilient and impenetrable to the sharpest needle.

The Degenerate Egg.

The behavior of the over-ripe ovum is extremely interesting and instructive in its bearing on the mechanism of the egg contents. As already stated, an ovum ultimately gelates and usually disintegrates. A gelated egg may be torn apart with great ease. If hardening has progressed far enough the egg may be cut in pieces as one would cut butter. If, on the other hand, the contents are not too solid the matrix may be readily drawn out into a long invisible thread. It is this gelated cytoplasmic matrix which is water-miscible.

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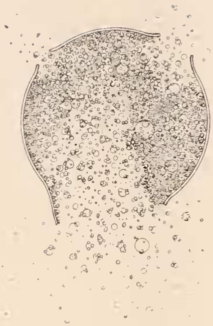

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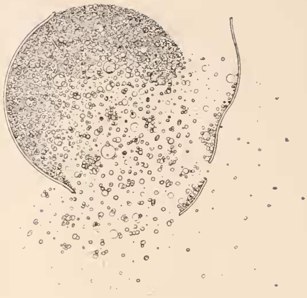

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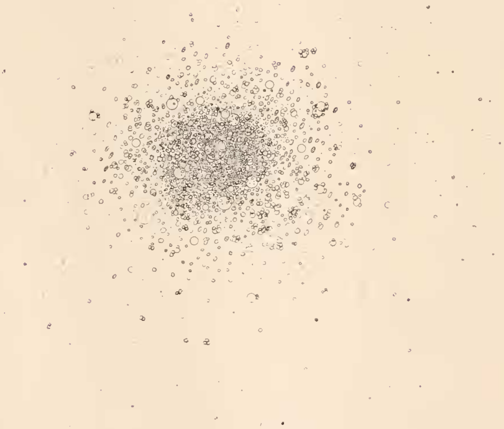


DESCRIPTION OF DRAWINGS.

FIG. 1. A disintegrating *Fucus* ovum in which smaller craters have burst forth subsequently to several seconds of activity on the part of the larger crater.

FIG. 2. A degenerate *Fucus* ovum in which discarded fragments of the wall are seen, and in which dissemination of granules is very active in one region while non-existent in another.

FIG. 3. The wild and rapid scattering of the inclusions of an exploded *Fucus* egg.

The disintegration of a degenerate egg is accomplished by the matrix going into solution with the sea-water. This may take place with such rapidity as to suggest a miniature explosion of the egg; or may be delayed and then it usually proceeds slowly, either in spasmodic periods of dissolution, or continuously until the entire granular contents has been disseminated. Often this is never completed. Where the disintegration is instantaneous (Fig. 3) it takes place immediately on the over-ripe egg coming in contact with a needle, or by the egg itself before actual dissection can be got under way. The miscibility of the matrix may be general (Fig. 3), or localized (Fig. 1). Where dissolution takes place without dissection some internal pressure, evidently osmotic, brings about a rupture. But it is difficult to see why such a force does not greatly expand so pliable a wall before breaking it. This is not the case. Furthermore, where disintegration has been instantaneous wall fragments are not to be found. When the process is gradual bits of it are only occasionally seen (Fig. 2). There is some reason for believing that the pliable, glutinous wall itself undergoes a change during degeneration of the protoplasm. If it does not go into solution it probably becomes a soft, inelastic gel.

A distributed, internal, osmotic pressure would be relieved once the egg-wall had broken at any one point, and one would not expect further eruptions. Quite the contrary is true. There may be a rather general dissemination of granules from one region for several seconds, time enough to relieve any pressure within, and subsequently, other craters break forth as shown in Fig. 1. These are localized centers of activity. That scattered regions of the egg contents are in different physiological states is further evident from the fact that certain localities may from the very outset remain inactive, never taking part in the general dissolution of the protoplasm, such as the exposed upper surface in Fig. 2.

The force responsible for a wild scattering of the inclusions in an ovum is a surface-tension one, due to an extraordinarily rapid miscibility of the degenerate matrix in the surrounding medium of sea-water.

ECHINARACHNIUS.

Microdissection work on the egg of the sand-dollar was undertaken for the purpose of comparison with the behavior of the plant ovum.

The difference in structure and behavior of *Fucus* and *Echinarachnius* ova before fertilization is not great. With fertilization two striking dissimilarities originate. There is in *Echinarachnius* an increase in viscosity of the protoplasm after fertilization. The path of a needle closes very slowly. Furthermore, there is very little change in the character of the egg wall. The wall of the young embryo is of no greater thickness than that of the ovum and but slightly more resistant.

The *Echinarachnius* egg occasionally tolerates a very great amount of dissection before completely deteriorating. At times, the capacity for wall formation persists in a region of still liquid plasma even after other regions of the egg have become an exceedingly viscous mass. This illustrates further the great diversity in the behavior of the protoplasm of eggs existing under apparently identical environments, and the marked difference in physiological condition of different regions of the same cell.

During the dissection of more than 20 eggs of the sand-dollar but one instance of miscibility was observed, and this was the rapid dissemination of granules due to the going into solution of the matrix of a gelated, that is, a degenerating ovum.

DISCUSSION.

In spite of the variety of objects studied and the differences in behavior of very similar material there are certain definite properties which characterize all the protoplasm here observed.

Streaming protoplasm is of a very liquid consistency. The same is true of young, actively growing plasma such as that found in the developing oögonium and the embryo of *Fucus*. The increase in consistency of the *Echinarachnius* ovum following the entrance of the sperm is not in harmony with this fact, nor is it what one would expect; for the liquid condition of protoplasm enables the elaborate chemical reactions of an active cell to take place. In the ripe egg, awaiting fertilization, metabolism is reduced to a minimum, so that a liquid state is not needed as it is for the complex chemical activities and interchange of substances which go on in the developing egg and growing embryo. In accordance with this is the fact that normal passive protoplasm in mature eggs of *Fucus* and *Echinarachnius* ova is quite viscous. (In ripe and resting seeds the protoplasm becomes almost solid.)

There is no reason for believing that any living protoplasm is naked. The surface membrane of protoplasm is at all times a definite morphological structure. The capacity to form such a pellicle is one of the characteristic properties of the living substance and is retained to a very late stage in dying protoplasm.

The membrane formed in repairing a tear is of the same character as the original. This is not true in those instances where the wall is of cellulose, as in pollen-tubes and plant embryos; for the enclosing surface layer of protoplasm is but a transformed portion of the living substance, the result of an immediate conversion of liquid plasma into a rigid gel of greater molar concentration. (Pfeffer, 1891, p. 194). It is worthy of note that protoplasm possesses not only the capacity to form a membrane but that kind of a membrane characteristic of a particular organism or of a particular stage of development. This suggests that the process is to some degree controlled from within.

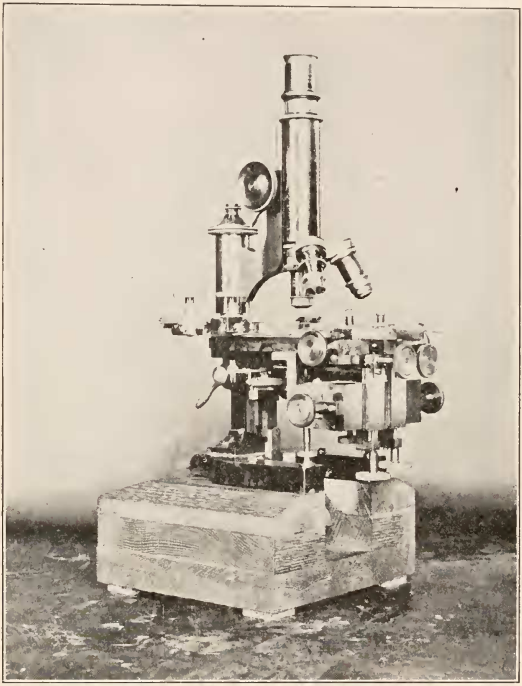
That contact with a *certain* medium is not necessary for membrane formation is evidenced from the fact that streaming myxomycetes form membranes whether dissected on a dry coverslip or in a hanging drop, and that the pellicle of the *Fucus* ovum is repaired whether it is torn open in the jelly-mass of the oögonium or in sea-water. Thus does it appear that contact with some medium is not a prerequisite to membrane formation, the lack of a membrane being the stimulus. Membranes are formed on any free protoplasmic surface. The general belief is that this is a purely physical process, and owes its origin to surface forces. It is somewhat disturbing to this strictly mechanistic conception that the capacity for membrane formation ends with death. (Pfeffer (1877) has shown experimentally that a membrane may be formed after death. Chemistry could give us many such instances. In neither case is the membrane a natural one.) Surface forces certainly come into play, but the capacity for membrane formation, and beyond doubt the factor determining the kind of membrane formed, is dependent upon the physiological state of the protoplasm, which, in a sense, means that the ultimate control lies within the cell; though, of course, not beyond the purely physical and chemical properties of the living substance.

The prevailing idea of a cell wall is that it is a dead structure and, therefore, incapable of change or response to environment. This is probably true of the cellulose walls of older cells, but not of the distensible, glutinous membranes and egg-walls here discussed nor of the cell-walls of meristematic tissues in general. The suggestion that the wall of a degenerating ovum may and does undergo, with the egg-protoplasm, a change which permits either its going into solution or its ready rupture by internal osmotic pressure, is not without experimental support. Protoplasmic membranes are an intimate part of the living substance and susceptible to the same changes in environment. (Bayliss, 1916, p. 115.)

Normal protoplasm, in all cases studied by the writer, does not mix with water. This declaration is contrary to that of Chambers (1917*a*, p. 2). If the decision is to rest on evidence gained solely from microdissection then miscibility of the protoplasm is a consequence of degeneration. Chambers's statement, however, rests not only on microdissection but on the introduction of water into the cell by the mercury injection method as well. Miscibility precludes the presence of a membrane. Normal protoplasm is always capable of membrane formation. Therefore, normal protoplasm can at no time be miscible. The dissection of a great many eggs supports this conclusion.

To the highest powers of the microscope, with ordinary illumination, protoplasm is a homogeneous, structureless solution. Its colloidal nature becomes evident on using the dark ground illuminator. Protoplasm is probably an emulsoid hydrosol, *i. e.*, a colloid in which both phases are liquid, one of them—the dispersion medium—being water.

Any definite statement on the ultimate structure of protoplasm must be expressed in physicochemical terms, and based upon observations made with the ultra-microscope. The behavior of protoplasm under dissection, however, throws considerable light on the gross structure of the cell contents. In distinguishing between gross and ultimate structure the writer has in mind, under the former, a haphazard arrangement of the minute subdivisions or centers of activity, of the living substance which are at any one moment in differing physiological states; while an ultimate structure connotes a definite arrangement of molecules or of the infinitesimal particles seen through the ultramicroscope—the microns or inferred amicrons of which all colloidal systems are composed.



Description of Photograph.

A two-needle Barber pipette holder attached to a Leitz microscope.

The most remarkable feature of life within a cell is the coexistence of a great number of reactions, constantly going on without one interfering with the other. This demands the division of the contents into innumerable chambers or centers of activity. Thus does the protoplasm possess, in a sense, structure. The presence of these miniature laboratories is well illustrated by the behavior of disintegrating ova in which the dissemination of the inclusions is at times in certain regions extremely rapid, and in others non-existent (Figs. 1 and 2). The difference in the physiological state of the protoplasm at any one moment is responsible for the variety of behavior of eggs from the same oögonium and of parts of the same ovum. This difference is to be expected if protoplasmic activity takes place in pulsations, which are neither synchronous among the eight eggs of one oögonium nor rythmic in any single ovum. The cell is, then, a laboratory in which many different chemical reactions are constantly going on, kept free from one another by boundaries of some kind. (Hofmeister, 1901.)

Structure in protoplasm is secondary to activity. Upon the chemical nature of the substances does the life of the cell depend rather than on their arrangement. What physical structures may exist are of a transitory nature. This interpretation does not preclude the all-important organization upon which the continuance of these activities depends.

SUMMARY.

1. Protoplasm is an emulsion colloid normally in the sol state.

2. The density of protoplasm varies from the very liquid state of young *Fucus* oögonia and embryos and of streaming protoplasm in myxomycetes and pollen tubes, to the quite viscous condition found in mature and resting eggs of marine organisms.

3. There is a rapid increase in viscosity of the *Fucus* egg during the last stages of its ripening, which is, on fertilization, followed by a return to the liquid consistency characteristic of active, growing proptolasm.

4. The plasma membrane is a definite morphological structure, constantly and repeatedly capable of repair through the conversion of the fluid protoplasm into a hyaline layer of greater molar concentration. This film of gel is exceedingly elastic, pliable and glutinous.

5. The surface layer, like the interior cytoplasm, seems to be capable of alteration with changes in environment.

6. The capacity for membrane-formation is one of the last essential properties of the living substance to be lost. It is lost only at death.

7. The kind of membrane formed is apparently identical with the parent membrane (except in the case of escaped protoplasm from cells which possess a cellulose wall).

8. The formation of a membrane is probably a purely physical process, but is dependent upon the physiological condition of the protoplasm. It is not dependent upon the surrounding medium.

9. The amount of physical disturbance that protoplasm can be subjected to before showing signs of injury varies from that of the immature *Fucus* ovum, where it is exceedingly great, to that of the ripe egg where it is very slight, often no more than a touch sufficing to cause disorganization.

10. Gelation of the plasma always takes place in time and is hurried by dissection. It is accompanied by degeneration.

11. Normal protoplasm is at all times non-miscible in water. Miscibility of the plasma is an unfailing criterion of degeneration.

12. Dissolution of the *Fucus* ovum is the result of the disorganized cytoplasmic matrix going into solution with the surrounding water. This mixing may take place with the rapidity of an explosion, or slowly, and then either continuously or spasmodically.

13. The disintegration of the egg plasm is frequently localized, in that certain regions of the contents continue dissemination of the granules from the beginning, while others join in later, and still others never take part. This indicates a gross structure of the egg plasm, *i. e.*, the protoplasm is composed of many centers of activity in which different chemical reactions take place separated by protective partitions.

14. That any definite and permanent arrangement of the colloidal particles exists seems unlikely. Whatever structure, gross or ultimate, protoplasm may possess is secondary to chemical activity upon which the life of the organism depends.

The writer wishes to thank Professor Duncan S. Johnson for suggesting this study and for assistance during its progress.

To Dr. Robert Chambers the writer is indebted for helpful suggestions, and to Dr. E. V. Cowdry for the use of his Barber instrument.

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FURTHER STUDIES ON THE PRODUCTION OF FUNCTIONAL AND RUDIMENTARY SPERMATOZOA IN ROTIFERS.[1]

D. D. WHITNEY.

In a recent paper some general observations were recorded in regard to the dimorphic spermatozoa found in the parthenogenetically developed males of nine species of rotifers. Some of the stages in the development of the spermatozoa from the cells of the last spermatocyte-divisions in *Brachionus mulleri* were described and figured. No studies, however, were made on the nuclear material of the spermatozoa either to determine whether it was present in both kinds of spermatozoa or to determine in what part of the spermatozoa it was located.

Additional studies now have been completed in an attempt to observe these points. These studies have been made in considerable detail on the marine species, *Brachionus mulleri*, and in lesser detail on *Hydatina senta*, *Brachionus amphiceros* and *Diaschiza sterea*. As the earlier paper had some of the details of the formation of the spermatozoa of *Brachionus mulleri* and as this rotifer is one of the most convenient forms to maintain in the laboratory and also a very convenient one from which to obtain immature males the majority of the observations were made on material from this species.

Several killing and fixing fluids and stains were tried but Zenker's fluid followed by Delafield's hematoxylin proved to be the most satisfactory method for making clear mounts of the entire cells. The living males were crushed under a cover glass in culture water and favorable cells were selected under the microscope. The killing fluid was allowed to run under the cover glass and to remain from 10-20 minutes. Later the killing fluid was drawn out by filter paper and distilled water allowed to take its place. After the distilled water had been changed several times from under the cover glass Delafield's hematoxylin

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[1] Studies from the Zoölogical Laboratory, The University of Nebraska, No. 119.

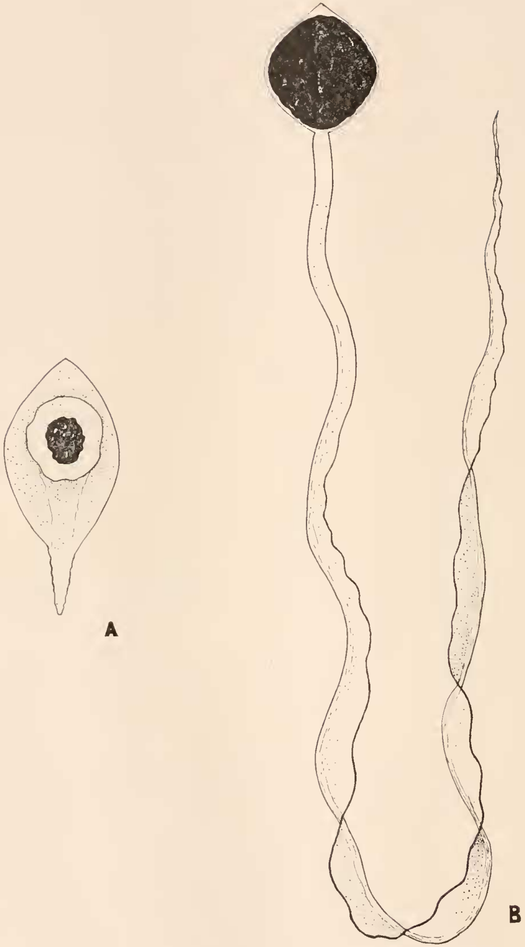


FIG. 1. *Brachionus mulleri.* *A*, normal spermatid showing the nucleus, chromatin, and the small tail; *B*, mature and normal spermatozoön showing the large chromatin mass in the head and the long vibratile tail with the undulating membrane. (Drawn by measurements from living and stained specimens with 1/12 oil immersion objective and a number 2 ocular.)

(diluted 1:1) was allowed to run under and to stain the cells for about two minutes. This was then washed out with tap water. All of these processes were carried on under the microscope in order to make certain that no mistakes of indentification of these cells should be made after the slides were finished. Although this is a tedious method it has the advantage of being perfectly reliable and furnishing accurate data concerning which there can be no doubts. Many other methods were tried but none were found to be as practical and as certain as this one.

The nucleus was first indentified in an early stage of the normal spermatid (Fig. 1, *A*) which later would have developed into the large motile spermatozoon (Fig. 1, *B*).

The degenerate sperm cells from the same male individual were examined next and each was found to contain a nucleus with chromatin material in it. This stage is shown in Fig. 2, *A*. This fact is of considerable interest because it demonstrates that in the division that forms the secondary spermatocytes there is evidently a division of the chromatin material and consequently all the secondary spermatocytes contain some of this chromatin material. One half of these secondary spermatocytes divide and form the normal spermatids but the remaining half of the secondary spermatocytes do not divide again but develop directly into the degernerate spermatozoa. The early stage of these degenerate spermatocyte cells are smaller in size than the normal cells of the same age but the nucleus is only slightly smaller than the nucleus contained in the normal spermatid cell as can be seen by comparing Figs. 2, *A* and 1, *A*.

The later development of each of these two kinds of cells is very different. In the normal cell the nucleus grows larger and larger until in its final stage it is several times its former size. From one end of the cell an outgrowth appears which grows longer and longer and finally becomes vibratile. This is the motile tail of the sperm cell. It is very long and large and has an undulating membrane along the greater portion of the dorsal side. Fig. 1, *B*, shows the entire matured spermatozoön containing the large mass of chromatin material in the head.

The development of the smaller and degenerate spermatocyte cells into the complete rudimentary spermatozoa is strikingly different from the development of the normal spermatid cells. After the early stage is passed the chromatin material increases in bulk very slightly and becomes composed of coarse granules

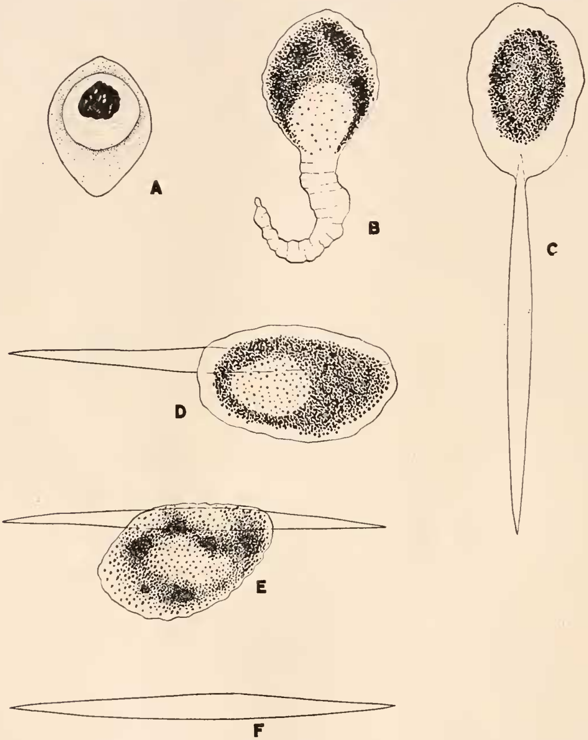


FIG. 2. *Brachionus mulleri*. *A*, early stage in the development of the rudimentary spermatozoön showing the chromatin in the nucleus. This cell is from the same male as the normal spermatid of Fig. 1, *A*, and is of the same age. *B*, a later stage showing the tail wrinkled and distorted by the fixing fluid; *C*, last stage showing the stiff and rigid tail attached to the head which contains the chromatin; *D*, the stiff tail forced through the chromatin to the opposite side of the head by pressure under the cover glass; *E*, the stiff tail forced through the opposite wall of the head by pressure under the cover glass; *F*, the stiff tail detached from the head. (Drawn to same scale as Fig. 1.)

loosely arranged and persists in this form throughout the remainder of the development of the cell. From one end of the cell a process extends which is somewhat flexible in its earlier development. Fig. 2, *B*, shows such an early stage in which the tail-like process has become curved and wrinkled after treatment with Zenker's fluid. Later this tail-like process becomes very rigid and stiff. So stiff that when the immature males are crushed this process by chance may be driven through the nuclear material to the opposite side of the cell as shown in Fig. 2, *C*, or in some instances it was even driven through the opposite wall of the cell as shown in Fig. 2, *D*. Normally this stiff process seems to be attached to one end of the cell. Later, however, when it is fully formed it becomes detached from the cell. This separation of the two parts of the cell occurs in the testis either before the male hatches from the egg or very soon thereafter. Fig. 2, *C*, shows the two parts just prior to separation.

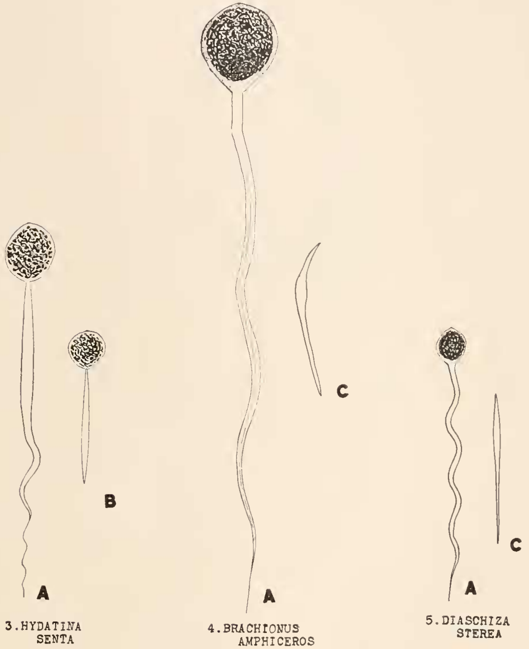
This stiff spindle-shaped part contains no chromatin material and probably is to be considered the rudimentary tail of the cell. It is immotile and much smaller than the tail of the motile spermatozoön. As it separates from the nucleated portion of the cell and is immotile one could naturally conclude that it is to be regarded as simply a degenerate or rudimentary tail which has no function. Whether the nucleated portion of the cell has any function is unknown but as it is also immotile it is also very probably functionless.

In the earlier studies the detached tails of the normal spermatozoa were erroneously considered to be the entire spermatozoa. When the males were crushed under a cover glass these tails were extruded and were able to move about in the water for a considerable length of time. When recently, however, these so-called spermatozoa were stained no nucleus or chromatin material could be found in any of them. As they are the functional spermatozoa it was realized that there must be a nucleus and chromatin material somewhere in them.

It was recalled that in previous observations some males had been seen to extrude a few of these motile bodies which had a large swelling on the anterior end. At that time these motile bodies were considered to be the immature spermatozoa. When more careful observations were made both upon such living cells and upon stained ones it was readily seen that these cells were not immature stages but were the mature stages of the spermatozoa. The heads are quite large in proportion to the diameter of the tail, Fig. 1, *B*, and are easily separated from the tails. In the testis of the male these spermatozoa are in clusters and in each cluster each spermatozoön is attached to some sort of a central tissue in the cluster. Normally in copulation with the young females a few of these spermatozoa become detached from the central tissue and are extruded into the body cavity of the female. When, however, the males are crushed under a cover glass nearly every head remains attached to this central tissue but the tails break off from the heads and are immediately extruded in a writhing mass. If the males are sufficiently crushed the mass of spermatozoa heads will also be extruded together with other cells of the broken-down tissues. If very young males are taken from the eggs and are crushed all of the immature sperm cells with the tails attached will be extruded. The ripe spermatozoa resemble very closely these immature spermatozoa except their heads are more rounded and the tails are somewhat longer.

The males of another rotifer, *Hydatina senta*, were crushed and the spermatozoa reëxamined. A few males shed both kinds of spermatozoa with the tails attached to the heads. These were stained and the heads of each kind were found to contain chromatin material similar to the heads of the spermatozoa of *Brachionus mulleri*. These are shown in Fig. 3. Twenty-five males were crushed and out of the many hundred spermatozoa ejected only about 6–8 entire spermatozoa of each kind with the tail attached to the head were found. *Hydatina senta* males are the most favorable material thus far examined in which to see the two kinds of complete spermatozoa in a mature male. In *Brachionus mulleri* these two kinds of complete spermatozoa could only be found in the unhatched and immature males.

The spermatozoa were examined from mature males of two additional species of rotifers, *Brachionus amphiceros*, and *Diaschiza sterea*. The entire normal spermatozoa with the tails attached to the heads were found in each species. Upon staining the heads showed their chromatin contents. Only the tails of the rudimentary spermatozoa were found and they showed no chromatin material in them when stained. Sketches of these spermatozoa and tails are shown in Figs. 4 and 5.



FIGS. 3–5. Dimorphic spermatozoa and tails of three other species of rotifers. *A*, larger normal and motile spermatozoa showing the head and the contained chromatin; *B*, smaller, rudimentary and non-motile spermatozoön showing the chromatin in the head; *C*, detached tails of rudimentary spermatozoa. (Drawn with 4 mm. objective and number 2 ocular.)

The spermatozoa of the other species of rotifers enumerated in the former paper have not been reëxamined because these forms are not available at this season of the year.

As the stained slides of the sectioned unlayed fertilized eggs of *Hydatina senta* were available for study they were examined to determine the size and condition of the head of the spermatozoön after it had entered the egg. The egg examined was sectioned while inside the body of the female and although it had the thick secretion around it which later would have shrunken to form the heavy external covering or shell it was readily sectioned at this early stage. The sperm head is seen near the nucleus of the egg (Fig. 6). It is about the size of the head of the normal spermatozoön as can be seen by comparing it with Fig. 3, but contains denser chromatin material and is perfectly round in form.

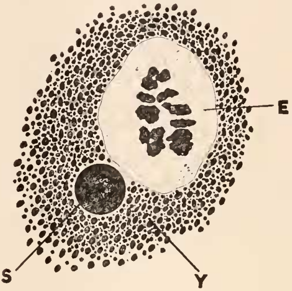


FIG. 6. Portion of a fertilized egg of *Hydatina senta*. *E*, egg nucleus containing chromosomes; *S*, sperm-head; *Y*, yolk granules of the egg. (Sperm-head and the outline of egg nucleus drawn to same scale as in Figs. 3–5. Yolk granules and chromosomes drawn freehand without measurements.)

Dimorphic spermatozoa have been studied in considerable detail in some of the Mollusca and in some of the Lepidoptera by several workers. Among these Goldschmidt and Gatenby recently have come to the conclusion that the atypical or abnormal spermatozoa are wholly functionless and the latter is of the opinion that such spermatozoa have no significance in regard to sex regulation. According to their conclusion any spermatozoa and in some cases all spermatozoa of these forms studied may become degenerate if the male individual is in a certain physiological condition.

In the phylloxerans and in the rotifers the case is quite different. In each and every male a certain percentage of the sperm cells degenerate and a certain percentage of the sperm cells develop normally, thus giving a definite ratio of degenerate and normal sperm cells. In the phylloxerans this ratio has not been determined but in the rotifers there is one degenerate sperm cell to two normal sperm cells. As all the fertilized eggs in both phylloxerans and rotifers develop into female young it seems safe to conclude, as Morgan has already concluded, that the degenerate sperm cells are the male-determining ones and that the normal sperm cells are the female-determining ones.

SUMMARY.

1. The normal and motile spermatozoa of *Brachionus mulleri*, *Brachionus amphiceros*, *Hydatina senta* and *Diaschiza sterea* possess large heads in which is located the chromatin material.

2. The motile bodies of seven other species of rotifers which were figured and considered as the normal spermatozoa in the former paper were probably only the tails of such normal spermatozoa.

3. The degenerate and immotile spermatozoa as seen in *Brachionus mulleri* and *Hydatina senta* also possess heads in which there is chromatin material.

4. The stiff processes, called rudimentary spermatozoa in the former paper, are now considered to be not the complete rudimentary spermatozoa but only the degenerate tails of these rudimentary spermatozoa.

5. The sperm head in the fertilized egg of *Hydatina senta* is about the same size as the head of the normal spermatozoön of this species.

6. The four species of rotifers enumerated above together with the seven other species described in the former paper constitute eleven species of rotifers in which both normal and rudimentary spermatozoa have been found to occur.

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THE UNIVERSITY OF NEBRASKA,
LINCOLN, NEBRASKA, January 5, 1918.

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BIOLOGICAL BULLETIN

EFFECT OF ENVIRONMENT UPON INHERITED CHARACTERS OF *HYDATINA SENTA*.

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Introduction.

Several years ago it was discovered (Shull, 1915) that two distinct parthenogenetic lines of the rotifer *Hydatina senta*, one from England, the other from Nebraska, differed in certain physiological (and perhaps structural) characters: (1) the English line laid smaller eggs than the Nebraska line; (2) the English line habitually laid a large percentage of its eggs attached to the surface film of the water, while the Nebraska line laid most of its eggs at the bottom or sides of the vessel; (3) the eggs of the English line required longer to develop than did those of the Nebraska line; (4) and when the rotifers were killed in Bouin's fluid the foot was seldom, or only slightly, retracted in the English line, but considerably retracted in the Nebraska line.

When crosses were effected between these two lines, the F_1 lines and F_2 lines were all indistinguishable from the English line in all the above-named characteristics. It seemed as if segregation and recombination had failed, and that in some way the four characters were rigidly associated one with another.

At first it was regarded as possible that the four characters were not really distinct, but were different manifestations of a single (physiological) character. That character might have been a greater permeability of the cells in one line than in the other. Thus, if the Nebraska line were more permeable to oxygen, the increased metabolism might make its eggs larger. For the same reason the eggs of the Nebraska line might develop in less time. In like manner it might be that the Nebraska rotifers, able to get the required amount of oxygen at the bottom of the dish where the oxygen in solution was less abundant, lived most of the time at the bottom and laid their eggs there; whereas the less permeable English rotifers were forced to swim to the surface where dissolved oxygen was presumably more abundant, and laid eggs at the surface film. And if the foot muscles of the Nebraska line were more permeable to the killing fluid than were those of the English line, the greater contraction of the muscles of the former might thereby be explained.

It was possible to test the correctness of the above assumptions, in part, by artifically altering the expression of the inherited characters through changes in the environment. A number of experiments were performed to this end. However, before they were completed, an F_3 generation was obtained in which the association of the four inherited characters was broken. In this and the succeeding generations each one of the four characters was separated at least once from the others with which it was associated in the original lines.

Thus was proven that the four characters were not merely different expressions of one character. The experiments designed to test their separateness or singleness were, therefore, not completed, and were not published. It has now become necessary, however, to refer to certain of the results, and they are here described in the incomplete form in which they were left. Along with them are several experiments on the viability of fertilized eggs, as affected by external conditions. These are of interest to the experimenter from a practical standpoint, and also in relation to popular ideas concerning the fertilized eggs of *Cladocera.*

EXPERIMENTS.

Effect of oxygen upon the laying of eggs at surface and bottom of water.

On each of the dates named in Table I., approximately equal numbers of females of *Hydatina* were placed in two dishes. In one was placed water oxygenated by vigorously shaking it in an atmosphere having a high percentage of oxygen. The dish was then set, uncovered, under a bell jar in which was confined an atmosphere containing the same high percentage of oxygen as that with which the water was first saturated. Since the bell jar was being used daily for other experiments, the percentage of oxygen used was not always the same. From May 27 to June 22, and on July 8 (see Table I.), the atmosphere contained 40 per cent. of oxygen; on all other dates 60 per cent.

In the other of the two dishes was placed untreated water. This dish was also set under a jar sealed at the edges, to prevent excess of evaporation, but in ordinary air.

After 20 to 40 hours the dishes were removed and the number of eggs at the surface film and at the bottom counted. As shown in Table I., the eggs are much less abundant at the surface film in the presence of much oxygen than in air.

Table I.

Showing the Number of Eggs Laid at the Surface Film and at the Bottom of the Dish by Rotifers Placed in Air and in an Atmosphere Containing an Excess of Oxygen.

Date.	Air.		Excess of Oxygen.	
	Number of Eggs at Surface.	Number of Eggs at Bottom.	Number of Eggs at Surface.	Number of Eggs at Bottom.
May 27	12	5	1	15
29	37	48	16	55
June 3	22	22	16	27
22	2	42	0	25
28	62	20	19	49
29	8	25	7	29
30	43	85	18	121
July 1	38	19	17	27
2	40	33	34	39
3	45	6	14	41
4	42	36	23	76
6	12	5	8	22
8	23	10	12	22
Total	386	356	185	548
Percentage at surface	52.0		25.2	

Effect of oxygen upon the size of parthenogenetic eggs.

Eggs laid by rotifers in oxygenated water, and in untreated water, were obtained in the following manner. Three or four young females, due to begin egg laying in 6 to 12 hours, were placed in each of two watch glasses. One lot was immersed in water saturated with an atmosphere of which 40 per cent. or 60 per cent. was oxygen, and then set under a bell jar in a similar atmosphere. The other lot was placed in spring water, and the dish was set under a closed bell jar in air. After 24 hours the watch glasses were removed, and all the eggs that were in a horizontal position were measured by means of a screw micrometer eye-piece. The measurements are given in units of the scale. They are directly comparable with the measurement of eggs in my former paper (Shull, 1915) since all the measurements were made with the same microscope and with the same lenses.

The mean length, mean breadth, standard deviation, etc., of the two lots of eggs are given in Table II.

TABLE II.

A Comparison of the Eggs Laid by the Rotifer Hydatina Senta in Oxygenated Water and Untreated Water.

	In Oxygenated Water.	In Untreated Water.	Difference.
Number of eggs measured......	100	100	0
Mean length of eggs...........	16.208 ± 0.028	16.098 ± 0.027	0.110 ± 0.039
Mean breadth of eggs.........	14.054 ± 0.021	14.021 ± 0.018	0.033 ± 0.028
Standard deviation of length...	0.409 ± 0.019	0.404 ± 0.019	0.005 ± 0.026
Standard deviation of breadth..	0.308 ± 0.015	0.266 ± 0.013	0.042 ± 0.019

The eggs in the oxygenated water were a trifle larger than those in the untreated water, though it can hardly be said that the difference is statistically significant. However, a difference that cannot be proven by statistical treatment to be significant is not necessarily insignificant. It seems to me not improbable that the difference in length is significant, but it is very small in comparison with the difference between English and Nebraska eggs described in my earlier publication (*op. cit.*).

Effect of oxygen upon the time of development of parthenogenetic eggs.

A number of egg-laying females were put into each of two dishes in the evening. In one dish was placed ordinary water, in the other water that had been oxygenated in the manner described in the preceding experiments. The females were removed after about an hour, but the eggs which they had laid were left in the dishes. The oxygenated water in one dish was then removed and replaced with fresh oxygenated water, and the dish was set under a bell jar in an atmosphere containing an excess of oxygen (40 or 60 per cent.). To insure that mechanical disturbance or accumulation of metabolic products did not affect the time of development of the eggs unequally, the water in the control dish was also drawn off after the rotifers were removed and replaced with fresh water. This dish was then set under a bell jar in air.

The next morning the two dishes were examined at intervals of 20 to 30 minutes, and the young rotifers removed and counted as they hatched. In this way the approximate time of development of the eggs was determined. In Table III. these times are collected in groups, to the nearest half hour. When these figures are treated statistically, they compare with one another as in Table IV.

Table III.

Showing the Time Required for Development of Eggs in Oxygenated Water and in Untreated Water.

	Number of Eggs Hatching in (Hours).						
	11.5.	12.0.	12.5.	13.0.	13.5.	14.0.	14.5.
Oxygenated water	7	10	15	23	9	1	
Untreated water	7	8	12	15	14	4	1

Table IV.

Showing the Time of Development of Eggs in Oxygenated Water and in Untreated Water. Statistical Treatment of Data in Table III.

	In Oxygenated Water.	In Untreated Water.	Difference.
Mean time of development in hours	12.654 ± 0.104	12.803 ± 0.129	0.149 ± 0.165
Standard deviation of time of development	1.239 ± 0.074	1.497 ± 0.091	0.258 ± 0.117

The eggs in oxygenated water hatched in a trifle shorter time, and somewhat more uniformly, though the difference in each case is so small that it may be insignificant. The greater uniformity of the time of development in oxygenated water (second line of Table IV.) is not improbably significant.

Effect of Oxygen upon the Contraction of the Foot Muscles.

Rotifers were placed in water saturated with an atmosphere containing 40 per cent. of oxygen, under a bell jar containing a similar atmosphere, and kept there 24 hours. They were then removed and promptly killed in Bouin's fluid. Other rotifers from the same families were kept in ordinary water, under a bell jar in air, for 24 hours, then killed in Bouin's fluid.

The contraction of the foot muscles was noted in accordance with the following arbitrarily chosen degrees of contraction: (0) foot fully extended; (1) foot slightly contracted, toes bent to one side, but still visible; (2) foot considerably contracted, toes wholly concealed, but contraction limited to small region near toes; and (3) foot greatly contracted, region of contraction much greater than in preceding class. It is to be noted that this classification is not the same as that proposed for the English and Nebraska rotifers in my former paper (Shull, 1915). The descriptions there given were not applicable to the rotifers used in these experiments.

The degrees of contraction of the foot muscles of the two lots of rotifers is tabulated in Table V., and the statistical comparison of the figures in Table VI.

Table V.

Showing the Degree of Contraction of the Foot Muscles of Rotifers Kept in Oxygenated Water, and in Untreated Water, and Then Killed in Bouin's Fluid.

Degree of Foot Contraction.	Number of Rotifers of Given Foot Contraction.	
	In Oxygenated Water.	In Untreated Water.
0	35	21
1	97	111
2	64	64
3	2	2

Table VI.

Statistical Comparison of Data in Table V.

	In Oxygenated Water.	In Untreated Water.	Difference.
Mean foot contraction.........	1.16 ± 0.034	1.27 ± 0.030	0.11 ± 0.046
Standard deviation of foot contraction.....................	0.64 ± 0.021	0.71 ± 0.024	0.07 ± 0.031

The statistical treatment is based on the assumption that each degree of contraction of the foot muscles differs from the degrees next to it by unity.

As shown in Table VI. there is less contraction of the foot muscles in oxygenated water than in untreated water, but the difference is rather small compared with its probable error and in view of the crudity of the method of measurement may be without significance.

Effect of Temperature upon the Laying of Eggs at Surface and Bottom.

Equal numbers of rotifers were placed in a number of dishes of water. Some of the dishes were kept at room temperature which was fairly constant at 20° to 22° C. Other dishes were kept much cooler by setting them on a window sill outside. A thermometer was kept beside the latter dishes, and was frequently read. In Table VII., which gives the number of eggs laid at the surface film and at the bottom, the stated temperature

Table VII.

The Number of Eggs Laid at the Surface Film and at the Bottom at Room Temperature and at Considerably Lower Temperatures.

Date.	Room Temperature.		Low Temperature.		
	Number of Eggs at Surface.	Number of Eggs at Bottom.	Number of Eggs at Surface.	Number of Eggs at Bottom.	Temperature in Degrees C.
Nov. 28	44	74	1	6	8
Nov. 29	20	59	0	14	7
			0	8	
			0	9	
			0	9	
Dec. 3	8	20	0	6	4
			0	10	
			0	4	
			0	2	
			0	0	
Dec. 4	15	22	0	0	2
			0	1	
			0	1	
			0	0	
Dec. 7	74	20	0	3	3
			0	1	
			0	1	
Dec. 8	6	20	3	7	12
			2	19	
			0	0	4
			0	3	
Totals	167	215	6	104	
Percentage at surface	43·7		5·5		

of these cool dishes is an estimated average. Because fewer eggs were to be expected at such low temperatures, several dishes were kept at low temperature for every one at room temperature.

There is no question here that the eggs are laid more largely at the surface when the temperature is high. The experiment was repeated on a small scale with the same result.

Effect of Temperature upon the Viability of the Fertilized Eggs.

Freezing.—Freezing of fertilized eggs was designed as a practical measure only, in order to induce those eggs to hatch which would not otherwise hatch. The attempt failed, however, as shown by the following tests.

In the first experiment about 244 eggs that remained unhatched after hatching had ceased for 12 days in the lot of eggs to which they belonged, were divided into two approximately equal groups. One group was frozen over night by immersing, in a closed vessel, in a brine-ice mixture and the other was kept in water at ordinary temperature. Of the frozen lot none hatched in 19 days thereafter. Of the control lot at room temperature, one hatched in 10 days after the beginning of the experiment, none thereafter Freezing did not facilitate the hatching.

In the second experiment about 230 eggs that remained unhatched for 14 days after hatching of the eggs in the same lot had practically ceased were similarly divided into two groups, one of which was frozen over night and the other kept at room temperature. None of these eggs in either lot hatched in 19 days, after which time observation ceased.

From these experiments it appears that fertilized eggs not ordinarily capable of hatching can not be made to hatch by freezing.

Low Temperature Above Freezing.—Two lots of fertilized eggs from the same source and of approximately equal numbers were kept at different temperatures from the time they were laid until hatching was nearly complete. The eggs were laid between November 29 and December 8. One lot was kept at room temperature. The other was set outside on a window sill where daytime temperature, as shown by a thermometer placed beside the dishes, ranged from 2° to 12° C. The air temperature was much below this, but was moderated in the location of the eggs by a steam radiator near the window sill inside. It appears that the water in which the eggs were placed never froze and hatching occurred during this period of low temperature. From December 22 to December 25 the atmospheric temperature was considerably above freezing but was much colder thereafter. On January 3 the "cold" dish was removed to room temperature, but hatching had been nearly completed before that time. Table VIII. shows the number of eggs that hatched.

Table VIII.

Showing the Effect of Room Temperature and Lower Temperatures Upon the Hatching of the Fertilized Eggs of Hydatina Senta.

Date of Beginning Experiment.	Warm.			Cold.		
	Number of Eggs.	Date of Hatching.	Number Hatched.	Number of Eggs.	Date of Hatching.	Number Hatched.
Nov. 29....	44	Dec. 6	1	43	Dec. 23	1
Dec. 2....	10	Dec. 7	1	10	Dec. 23	2
					Dec. 24	2
					Jan. 5	1
Dec. 8....	43	Dec. 10	2	44	Dec. 10	1
					Dec. 12	2
					Dec. 24	1
Total number hatching..........			4			10

Low temperature appears to favor the hatching of the eggs, not as an after effect, but during the period of low temperature.

Effect of Oxygen upon the Viability of the Fertilized Eggs.

Each of four lots of eggs from a single source was divided into two equal parts. One was placed in water of high oxygen content (saturated with an atmosphere of which either 40 per cent. or 60 per cent. was oxygen) and set under a bell jar enclosing an atmosphere containing the same proportion of oxygen as that with which the water was originally saturated. Atmospheres of 40 per cent. oxygen were used until July 7, 60 per cent. thereafter. This dish was removed from the bell jar daily and examined for hatching rotifers. The water was drawn off the eggs after examination and replaced with fresh oxygenated water and the dish returned to the bell jar.

The other dish was filled with untreated water which was drawn off daily and replaced with fresh water.

The hatching of the eggs is recorded in Table IX.

TABLE IX.

Showing the Effect of Oxygenation of the Water Upon the Hatching of the Fertilized Eggs of Hydatina Senta.

Experiment.	Date of Starting Experiment.	Oxygenated Water.			Untreated Water.		
		Number of Eggs.	Date.	Number Hatching.	Number of Eggs.	Date.	Number Hatching.
A.......	July 1, 1915	24	July 3	1	24	July 2	1
			7	1		7	1
			9	1		8	2
						12	1
				3			5
B.......	July 2, 1915	30	July 6	4	30	July 5	4
			10	1		8	1
			21	1		9	1
						10	1
						17	1
				6			8
C.......	July 4, 1915	79	July 5	1	78	July 8	1
			9	4		9	5
			10	5		10	4
			11	2		11	1
			12	3		12	3
						13	1
						14	1
				15			16
D.......	July 7, 1915	100	July 10	1	100	July 8	1
			11	2		9	1
			12	2		10	1
			12	5		11	3
			14	2		12	1
						13	8
						14	3
						21	1
						23	1
				12			20
Grand total....	....			36	....		49

The eggs in oxygenated water show a somewhat lower viability in every case.

Effect of Drying upon the Viability of the Fertilized Eggs.

Dried for a Short Period.—A lot of cross-fertilized eggs—eggs laid by females of line B fertilized by males of line A of my former experiments (Shull, 1913)—were kept for seven weeks to allow hatching to take place. Practically all the hatching occurred in the second to fourth weeks of this period, almost none in the last three weeks. The 446 eggs (about two thirds of the original lot) which remained unhatched after seven weeks were divided into two nearly equal groups. One half was allowed to become dry December 8 and remain dry about 13 hours, after which it was remoistened. The other half was kept wet. The subsequent hatching of eggs from these two groups is recorded in Table X.

TABLE X.

Showing the Effect of Drying Eggs for a Short Period Upon the Proportion that Hatch in a Cross-Fertilized Lot of Eggs of Hydatina Senta.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Dec. 9	0		
10	0	Dec. 10	0
11	1	11	0
12	0	12	0
13	0	13	2
14	0	14	3
15	0	15	1
16	0	16	8
17	0	17	4
18	1	18	5
19	0	19	4
20	0	20	7
Total.	2		34

Observations necessarily stopped December 20, but it seems likely that even more would have hatched among the eggs that were dried. Drying for a short period either favors hatching of eggs that would not otherwise have hatched or hastens the hatching of eggs whose hatching would otherwise have been spread over a long period.

The above experiment was repeated with a lot of eggs from the reciprocal cross of the foregoing—eggs laid by females of line A fertilized by males of line B. The original lot of eggs was kept seven weeks to allow of hatching. During the last three weeks of that time very little hatching occurred. The 238 eggs that remained (less than half of the original lot) were divided into two equal parts, one of which was allowed to become dry and remain so for 13 hours on December 4. The subsequent hatching is recorded in Table XI. The results confirm the conclusions drawn from Table X.

TABLE XI.

Showing the Effect of Drying Eggs for a Short Period Upon the Proportion that Hatch in a Cross-Fertilized Lot of Eggs of Hydatina Senta. The Cross was the Reciprocal of that in Table X.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Dec. 5–20	0	Dec. 7–15	0
		16	1
		17	1
		18	16
		19	2
		20	1
Total.	0		21

In another experiment of this kind inbred eggs (eggs laid by females fertilized by males of the same line) of a line in which a relatively small proportion of the eggs normally hatched were used. The eggs were kept five weeks, during the last 12 days of which time no eggs hatched. The eggs that remained unhatched were divided into two lots which, by mistake, were made unequal. One lot, consisting of about 130 eggs, was dried over night; the other of 106 eggs, was kept wet. The subsequent hatching of these eggs is recorded in Table XII.

TABLE XII.

Showing the Effect of Drying for a Short Period Upon the Hatching of Inbred Eggs of Hydatina Senta.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Feb. 25 to Mar. 8 ..	0	Feb. 24 to Mar. 4 .	0
Mar. 9.	1	Mar. 5.	1
Mar. 10 to 14.	0	Mar. 6 to 14.	0

Unlike the cross-fertilized eggs of Tables X. and XI., drying for a few hours neither increased the number of eggs that hatched nor hastened their time of hatching.

The foregoing experiment was repeated with inbred eggs of a line that regularly hatched more than half its eggs. The eggs remaining unhatched after five weeks were divided into two lots, one of which was dried over night and then remoistened. Though both were kept three weeks, not one egg in either lot hatched. Drying neither increased nor hastened the hatching.

Dried for Periods of Moderate Length.—These experiments differed from the foregoing in that all of the eggs were dried in one half of the experiment, instead of only those which failed to hatch under ordinary conditions. Inbred eggs were used, and drying occurred about the time when hatching was due to begin, that is, a week after the eggs were laid. Hatching began three or four days after the eggs were remoistened. Observations were continued for a month after remoistening. One lot of eggs was kept wet as a control, one was dried over night, one dried two weeks, and a fourth dried four weeks. The experiment was performed three times. The totals, without daily records, are given in Table XIII. The second division of this table really belongs to the preceding section of this paper, since it involves only a short period of drying, but it seems best to retain it here for the sake of comparison.

TABLE XIII.

Showing the Effect of Drying for Various Periods Upon the Viability of the Fertilized Eggs of Hydatina Senta. Inbred Eggs Were Used.

Experiment.	Eggs Kept Wet.		Dried Over Night.		Dried Two Weeks.		Dried Four Weeks.	
	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.
A	75	40	78	35	76	18	72	0
B	55	26	41	6	50	12	53	0
C	39	23	33	19	38	6	27	0
Total.......	169	89	152	60	164	36	152	0
Percentage hatching ..	52.6		39.4		21.9		0.0	

There is plain indication in these results that drying, even for a short time, reduces the number of eggs that will hatch when again placed in water; and that the longer the period of desiccation, up to the limit of complete inhibition, the fewer the eggs that hatch.

Dried for a Long Period.—No experiment with control, involving a longer period of desiccation than four weeks, was performed; but that some eggs could withstand longer desiccation was shown. One lot of 344 cross-fertilized eggs and another of 100 inbred eggs, all from sources not used in the experiments described in this paper, were kept in dried condition from June 29 to March 23, or about nine months, when they were remoistened. In about three weeks thereafter three eggs of the first group and one of the second hatched.

All these eggs simply rested on the bottom of a watch glass when dried. It is not improbable that when the eggs, on drying, are supported by mud or sand they may remain desiccated longer and still hatch when remoistened. But even in mud the possible period of desiccation is not indefinitely long; for out of a lot of fertilized eggs in dried mud I have secured numerous young rotifers after three months of desiccation, but no eggs hatched from this lot after two years.

Summary and Discussion.

In former papers (Shull, 1913, 1915) the inheritance of size of parthenogenetic eggs, the time of development of parthenogenetic eggs, the place of laying parthenogenetic eggs (surface film or bottom), the viability of fertilized eggs (proportion that hatch), and the contractility of foot muscles was described. In this paper is shown to what extent these inherited characters may have been modified by such external agencies as temperature, oxygen, and desiccation.

It was found that if the water in which the rotifers live was exposed to an atmosphere containing more than the usual proportion of oxygen, a greater proportion of the eggs were laid at the bottom of the vessel. Under ordinary conditions the rotifers probably come to the surface because of the greater quantity of dissolved oxygen there. Those lines which normally lay their eggs mostly at the bottom probably either require less oxygen or get their oxygen more easily than lines which lay their eggs at the surface film. The hereditary character involved may therefore be the oxygen requirement or the permeability to oxygen.

If the rotifers were kept at a low temperature, their eggs were laid more largely at the bottom. This may be due to the greater concentration of oxygen in the water at low temperature, so that it is unnecessary to come to the surface so much; or to the low metabolism and hence low oxygen requirement of the animals; or to both of these reasons.

Excess of oxygen increased the size of the parthenogenetic eggs only very slightly, or not at all. Excess of oxygen decreased the time of egg development only very slightly, or not at all. It may have made the time of development a trifle more uniform, though this is hardly proven.

Rotifers kept in oxygenated water showed, when killed in Bouin's fluid, a trifle less contraction of the foot muscles than did other rotifers, though the difference was too small to constitute a proof of the action of oxygen.

Since the place of laying the eggs (surface or bottom) was so much more greatly affected by oxygen than were the other three characters tested, it would seem rather improbable even if the genetic results (Shull, 1915) had permitted such an assumption, that the several characters in which the English and Nebraska lines differ as described in the introduction to this paper, were really but a single character with several manifestations. The results described in this paper, therefore, harmonize with the genetic results previously obtained.

Freezing fertilized eggs that had remained unhatched for five to seven weeks did not induce any of them to hatch later. However, when eggs were kept from the time of laying at a low and variable temperature above freezing, more of them hatched than when kept at room temperature.

When fertilized eggs were kept in oxygenated water, a somewhat smaller proportion of them hatched than in untreated water.

Cross-fertilized eggs which had remained for seven weeks under ordinary conditions without hatching were dried over night and then remoistened. A considerable number of them were thereby caused to hatch. Inbred eggs, however, when tested in the same way, did not respond to drying. Even inbred eggs that were dried shortly after they were laid (not merely those which failed to hatch otherwise) did not show any increase in the proportion of viable eggs. Indeed, drying had just the opposite effect on inbred eggs. Even when the eggs were kept dry only a few hours, the percentage of them that hatched was reduced; and the longer the eggs were kept dried the fewer of them hatched. Those that remained dry for four weeks did not hatch at all.

Other lines were not as sensitive to drying, for out of one lot of eggs that were dry for nine months, several eggs hatched when remoistened.

In view of the results of desiccation of inbred eggs, it is conceivable that the hatching of cross-fertilized eggs after drying was due merely to the hastening of the development of eggs whose hatching would otherwise have been spread over a long period. If we had for comparison only the experiments with inbred eggs which were dried immediately after laying, and those with cross-fertilized eggs that were dried after they had been allowed abundant time in which to hatch and had not done so, the conclusion just stated would seem not merely conceivable, but probable. However, since inbred eggs were also dried after their normal hatching period was past, and failed to hatch subsequently, whereas cross-fertilized eggs thus treated did hatch, I am inclined to believe that drying for a few hours actually caused some cross-fertilized eggs to hatch which would not have done so without drying. From the physiological viewpoint, such a difference between inbred eggs and cross-fertilized eggs is not at all improbable.

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THE EFFECT OF SELECTION UPON THE SEX-RATIO IN DROSOPHILA AMPELOPHILA.

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FROM THE ZOÖLOGICAL LABORATORY OF INDIANA UNIVERSITY.[1]

It is a well-known fact that sex-ratios in most animals approximate equality. The ratio is seldom one of exact equality, however, and the preponderance may be in favor either of the males or the females. The variation from equality is fairly constant for the species. For man the ratio has been found to be 100 females to 105 males; for the horse, 100 to 98; for the cow, 100 to 107; for the sheep, 100 to 97; for the pig, 100 to 111; for the dove, 100 to 105; and for the hen, 100 to 94. These variations are so constant that they cannot be attributed to chance and they are irregularities for which our present theories of sex determination offer no adequate explanation.

The writer has attempted to determine the sex-ratio of the fruit fly, *Drosophila ampelophila*. The determination was made from three unrelated stocks in which the best possible environmental conditions were provided. All matings were in single pairs. Over 35,000 flies were examined and the ratio was found to be 100 females to 95 males.

Moenkhaus tested the effect of selection upon the sex-ratio of *Drosophila ampelophila* and decided that the sex-ratio in this species is "amenable to selection." If it be true that the relative number of males and females in a strain can be varied by selection, the present theories of sex determination must be somewhat modified. Although this fact would not necessarily disprove the theories of sex determination, it would necessitate the assumption of an hypothesis of selective fertilization or of differential mortality or viability of the determining elements.

In view of the vital bearing of Moenkhaus's findings upon the theories of sex determination, it has been deemed worth while to repeat his work.

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[1] Contribution No. 159.

Methods and Material.

The selection experiments were carried out upon three unrelated stocks. The stock for the first experiment was collected in Bloomington, Ind.; for the second, in Saratoga, Ind.; and for the third, in Warsaw, Ind. In the first two cases the experiments were started as soon as the stocks were collected from nature but the Warsaw stock was inbred in the laboratory for about six months before the experiment was started. At all times the greatest care was exercised to prevent contamination. The flies were provided with an abundance of food and care was taken to examine all the offspring of each pair. All matings were single pair matings.

Each experiment was conducted upon the offspring of a single pair of flies, that is, for the first experiment, a virgin female was selected from the Bloomington stock and mated to a brother. From among the offspring of this pair of flies, the parents of the two strains were obtained. The one strain in which there was selection for a relatively high number of females in comparison to the number of males, has been called the "high" strain and the one in which the selection was in the opposite direction, has been called the "low" strain. Before selection was started, a sufficient number of flies were examined to obtain a fair estimate of the sex-ratio of the stock under consideration. Moenkhaus's high and low strains were not offspring of a single pair of flies but were obtained by selecting from nature, pairs which had unusual ratios in the desired direction. Furthermore, he knew nothing of the original sex-ratio of his strains before selection began.

In each generation an attempt was made to examine the offspring of ten pairs in each strain. Unsuccessful matings, usually due to poor food conditions, sometimes prevented this. In each generation, the pair which gave the most extreme ratio in the desired direction was used as the parent of the succeeding generation. At times, technical difficulties prevented the most extreme pair being used and in these cases the next best was used. Pairs showing extreme ratios but producing a small number of offspring were not used. Since it was not possible to know which pair had the most desirable ratio till all of the offspring of all the pairs in a generation were examined, the technique of the experiment was made somewhat difficult. This might have been overcome by isolating some males and females from each mating and then, after the counts for that generation were completed, mating the offspring of the pair giving the most extreme ratio. This would have prolonged the experiment, so to avoid delay, at about the eighth or ninth day after the young had begun to emerge, fifteen pairs were mated from among the offspring of each mating which was being examined. In this way, by the time one generation was finished, the next was almost ready to begin to emerge. Since ten matings in each strain were examined, this necessitated the mating of about300 pairs of flies in each generation. A control was also carried in each experiment in which a few hundred flies were examined each generation.

Preliminary Experiment.

This experiment was to test whether the age of the parents would in any way influence the relative number of males and females produced. Several matings were made and the adults were transferred to new bottles every eight days as long as they both lived. The age of the parents does not influence the sex-ratio as is shown by the results given in Table I.

Table I.

Showing Results of Experiment to Test the Effect of Age of Parent Upon the Sex-Ratio.

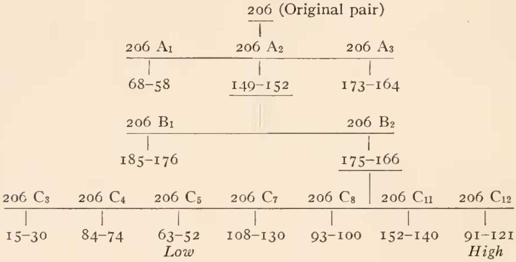
Culture Number.	Original Mating 1 Day Old.		First Transfer 11 Days Old.		Second Transfer 18 Days Old.		Third Transfer 26 Days Old.		Fourth Transfer 34 Days Old.		Fifth Transfer 42 Days Old.		Sixth Transfer 50 Days Old.		Seventh Transfer 58 Days Old.		Eighth Transfer 62 Days Old.		Totals.	
	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
$202A_2$.	56	68	112	110	105	111	51	40											324	329
$203A_2$.	93	92	110	128	60	89	126	124	172	152	49	60							610	645
$203A_3$.	156	162	135	150	58	60	78	89	136	123	91	93							654	677
$204A_1$.	232	194	140	146	20	24													392	364
$204A_2$.	223	234	155	156	157	151	115	115											650	656
$204A_3$.	134	128	46	70	38	37													218	235
$205A_2$.	214	202	109	114	86	104	115	110	12	12									536	542
$205A_3$.	187	161	140	130	103	113	114	107											544	511
$206A_1$.	68	58	80	55	75	77	78	76	94	89	72	95	126	118	45	45	10	5	648	618
$206A_2$.	149	152	173	176	79	126	135	106	152	130	34	23							722	713
$206A_3$.	173	164	179	207	141	134	129	162	156	159	183	203	71	91					1032	1120
$207A_1$.	90	87	153	143	81	90	100	100											424	420
$207A_2$.	120	118	129	123	86	83	84	69											419	393

SELECTION EXPERIMENT I. (Bloomington Stock.)

From a stock of flies collected in Bloomington, a virgin female was selected and mated to a male from the same stock. From the offspring of this pair of flies the high and low strains were started. Before selection was begun, 2,936 flies were examined for sex. The ratio was 1,456 males to 1,480 females, or 1 male to 1.06 females. The ratio here is stated in just the reverse order from the customary form but since Moenkhaus used the reverse order, the ratios in the present discussion will be stated likewise to make the two experiments more comparable. Table II. gives the history of the origin of the high and low strains.

TABLE II.[1]

Showing Origin of the "High" and "Low" Strains of the Bloomington Stock.



Tables III. and IV. give the history of the high and low strains. There is no indication of the ratios having changed in the direction of the selection. In fact, in the high strain where there was selection for a relatively high number of females, the totals show a relatively lower number of females than in the low strain. The totals for the high strain were 8,837 males to 8,942 females or a ratio of 1 male to 1.012 females while in the low strain there were 8,368 males to 9,091 females or a ratio of 1 male to 1.086 females.

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[1] In all tables where two numbers are expressed with a dash between them, as 68–58, the number to the left of the dash always represents the male count and the number to the right, the female count.

TABLE III.

Showing Summary of the "High" Strain of the Bloomington Stock. Experiment I.

Generation.											Totals.
A	91 ♂–121 ♀ (*1 : 1.33*) (From Mating 206C12)										
B	2 145–150 *1 : 1.03*	4 200–224 *1 : 1.12*	5 216–229 *1 : 1.06*	6 188–198 *1 : 1.05*	7 179–177 *1 : .99*	8 229–183 *1 : .80*	9 154–162 *1 : 1.05*	10 99–85 *1 : .85*	11 111–116 *1 : 1.05*	13 121–140 *1 : 1.16*	1642–1664 *1 : 1.013*
C	1 78–65 *1 : .83*	3 106–118 *1 : 1.11*	4 153–148 *1 : .97*	5 167–179 *1 : 1.07*	6 53–53 *1 : 1*	8 176–165 *1 : .94*	10 143–136 *1 : .95*	12 127–115 *1 : .91*		13 42–69 *1 : 1.64*	1045–1048 *1 : 1.003*
D	2 201–233 *1 : 1.16*	3 83–83 *1 : 1.0*	5 115–110 *1 : .96*	6 92–90 *1 : .99*	7 221–204 *1 : .92*	9 117–125 *1 : 1.07*	10 95–104 *1 : 1.09*	11 37–35 *1 : .95*	12 139–160 *1 : 1.15*	14 284–254 *1 : .89*	1384–1399 *1 : 1.01*
E	1 117–99 *1 : .85*	2 184–176 *1 : .96*	3 223–225 *1 : 1.01*	4 194–173 *1 : .89*	5 302–279 *1 : .92*	6 179–180 *1 : 1.01*	7 252–278 *1 : 1.1*	10 93–111 *1 : 1.19*	12 195–194 *1 : .99*	13 36–31 *1 : .86*	1775–1746 *1 : .984*
F	1 179–177 *1 : .988*	2 208–188 *1 : .90*	3 79–70 *1 : .89*	4 98–128 *1 : 1.31*	5 188–154 *1 : .82*	7 124–142 *1 : 1.15*	9 149–159 *1 : 1.07*	12 158–154 *1 : .97*	13 113–139 *1 : 1.241*	14 52–61 *1 : 1.18*	1348–1372 *1 : 1.018*
G	1 85–96 *1 : 1.13*	2 166–168 *1 : 1.01*	5 31–55 *1 : 1.77*	6 164–174 *1 : 1.06*	8 219–223 *1 : 1.02*	10 125–135 *1 : 1.08*	11 144–143 *1 : .99*	12 70–80 *1 : 1.143*	14 111–119 *1 : 1.07*	15 86–96 *1 : 1.12*	1201–1289 *1 : 1.073*
H	3 95–101 *1 : 1.06*			4 116–103 *1 : .89*		8 64–57 *1 : .89*	9 50–35 *1 : .70*	10 117–128 *1 : 1.09*			442–424 *1 : .959*

Grand Total........8837–8942
1 : 1.012

TABLE IV.

Showing Summary of the "Low" Strain of the Bloomington Stock. Experiment I.

Generation.											Totals.
A	63 ♂–52 ♀ (*1 : .83*) (From Mating 206C$_5$)										
B	1 164–228 *1 : 1.39*	3 227–248 *1 : 1.09*	4 109–113 *1 : 1.04*	5 138–146 *1 : 1.06*	7 190–187 *1 : .98*	8 35–88 *1 : 2.51*	9 185–184 *1 : .99*	10 191–197 *1 : 1.03*			1239–1391 *1 : 1.123*
C	1 123–135 *1 : 1.1*	4 51–53 *1 : 1.04*	5 291–327 *1 : 1.12*	7 105–119 *1 : 1.13*	8 120–114 *1 : .95*	9 60–44 *1 : .73*	10 176–181 *1 : 1.03*	11 52–71 *1 : 1.37*			978–1044 *1 : 1.067*
D	2 175–207 *1 : 1.18*	6 153–128 *1 : .84*	7 136–160 *1 : 1.18*	9 130–126 *1 : .97*	10 133–158 *1 : 1.19*	11 192–196 *1 : 1.03*	12 85–104 *1 : 1.22*	13 225–261 *1 : 1.16*	14 135–158 *1 : 1.17*	15 181–199 *1 : 1.2*	1545–1697 *1 : 1.098*
E	1 176–141 *1 : .8*	2 125–123 *1 : .98*	4 138–131 *1 : .95*	5 141–135 *1 : .96*	8 151–151 *1 : 1*	9 139–142 *1 : 1.02*	10 285–277 *1 : .97*	11 167–141 *1 : .84*	12 193–234 *1 : 1.21*	15 180–180 *1 : 1*	1695–1655 *1 : .976*
F	1 275–252 *1 : .92*	2 174–167 *1 : .96*	3 145–179 *1 : 1.23*	4 115–105 *1 : .91*	6 173–190 *1 : 1.10*	7 194–180 *1 : .93*	9 164–264 *1 : 1.61*	11 246–266 *1 : 1.08*	12 219–215 *1 : .981*	14 181–202 *1 : 1.11*	1886–2020 *1 : 1.071*
G	1 114–134 *1 : 1.18*		6 84–180 *1 : 2.14*		9 89–83 *1 : .93*	11 205–190 *1 : .93*	14 37–30 *1 : .81*	15 84–132 *1 : 1.57*			613–749 *1 : 1.221*
H	1 52–64 *1 : 1.23*	4 56–72 *1 : 1.29*	5 59–87 *1 : 1.47*	7 48–70 *1 : 1.46*	8 38–47 *1 : 1.24*	10 47–53 *1 : 1.13*	12 8–14 *1 : 1.75*	13 9–9 *1 : 1*	14 45–61 *1 : 1.36*	17 50–58 *1 : 1.16*	412–535 *1 : 1.298*

Grand Total........8368–9091
1 : 1.086

Selection Experiment II. (Saratoga Stock.)

In the first experiment, a much larger number of flies were examined than were examined by Moenkhaus. But since opposite results were obtained, a criticism might be offered that the writer, by chance, selected a stock which was not affected by selection. So the second experiment was made upon a new and unrelated stock. This stock was collected at Saratoga, Indiana. Fifty matings were made from among the offspring of a single pair. The extremes of these fifty were used as parents of the high and low strains. The counts of these fifty pairs are given in Table V. There were 21,019 flies examined before selection began and the stock showed a ratio of 1:1.0512, or 10, 247 males to 10,772 females.

Table V.

Showing the Origin of the "High" and "Low" Strains of the Saratoga Stock.

♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
264	**235**[1]	202	222	252	241	213	248	179	149
157	165	246	236	157	190	187	168	266	263
208	230	290	280	163	202	241	235	224	234
285	256	192	232	214	237	232	257	109	129
137	160	218	223	176	188	245	205	184	171
173	190	187	217	243	282	75	106	226	206
193	187	251	222	160	189	245	225	195	233
155	168	192	207	200	280	188	171	183	168
252	236	**181**	**307**[2]	212	275	129	175	226	222
288	271	183	187	200	225	235	247	234	220

Grand Total.............................. 10,247 ♂'s to 10,772 ♀'s

The results of the selection in this experiment are given in Tables VI. and VII. In this stock there were greater individual variations from the normal sex-ratios in each direction but they did not breed true. The totals show the high strain to have a higher relative number of females than the low strain. They are as follows: for the high strain, 7,377 males to 8,365 females, or a ratio of 1:1.134; for the low strain, 10,923 males to 11,246 females or a ratio of 1:1.029. Considering these totals, it might seem that something had been accomplished by selection but when the totals are considered generation by generation the results do not seem so conclusive. If selection has been the factor which

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[1] From this mating originated the "low" strain.

[2] From this mating originated the "high" strain.

TABLE VI.

Showing Summary of the "Low" Strain of the Saratoga Stock. Experiment II.

Generation.											Totals.
A			264 ♂–235 ♀ (*1 : .89*)								
	1	2	3	4	5	6	7	8	9	10	
B	146–150 *1 : 1.03*	159–202 *1 : 1.27*	199–152 *1 : .76*	199–213 *1 : 1.07*	131–129 *1 : .99*	83–91 *1 : 1.10*	81–78 *1 : .96*	109–119 *1 : 1.09*	108–100 *1 : .926*	107–121 *1 : 1.13*	1322–1355 *1 : 1.024*
	1	2	3	4	5	6	7		8	10	
C	144–161 *1 : 1.12*	135–143 *1 : 1.06*	210–208 *1 : .99*	132–130 *1 : .98*	138–149 *1 : 1.08*	157–138 *1 : .88*	224–251 *1 : 1.12*		45–44 *1 : .98*	90–84 *1 : .93*	1275–1308 *1 : 1.0259*
	1	2	3	4	5	6	7	8	9	10	
D	270–294 *1 : 1.09*	212–242 *1 : 1.14*	174–135 *1 : .78*	193–217 *1 : 1.12*	223–211 *1 : .95*	256–246 *1 : .96*	67–81 *1 : 1.21*	219–215 *1 : .98*	308–308 *1 : 1*	267–263 *1 : .985*	2189–2212 *1 : 1.0105*
	1	2	3	4	5	6	7	8	9	10	
E	137–136 *1 : .99*	106–115 *1 : 1.08*	50–65 *1 : 1.3*	146–169 *1 : 1.16*	115–130 *1 : 1.13*	153–153 *1 : 1*	109–138 *1 : 1.27*	206–188 *1 : .91*	32–39 *1 : 1.22*	160–149 *1 : .931*	1214–1282 *1 : 1.056*
	2		3		4		5	6			
F	166–176 *1 : 1.06*		199–213 *1 : 1.07*		72–84 *1 : 1.17*		171–210 *1 : 1.23*	62–37 *1 : .60*			670–720 *1 : 1.0746*
	1	2	3	4	5	6	7	8	9	10	
G	43–41 *1 : .95*	101–97 *1 : .96*	130–137 *1 : 1.05*	124–121 *1 : .98*	79–90 *1 : 1.14*	154–170 *1 : 1.10*	90–103 *1 : 1.14*	148–156 *1 : 1.05*	68–67 *1 : .985*	205–213 *1 : 1.039*	1142–1195 *1 : 1.0464*
	1	2	3	4	5	6	7	8	9	10	
H	199–197 *1 : .99*	172–169 *1 : .98*	144–152 *1 : 1.06*	169–179 *1 : 1.06*	151–152 *1 : 1.01*	124–141 *1 : 1.14*	92–101 *1 : 1.10*	210–225 *1 : 1.07*	163–185 *1 : 1.135*	114–107 *1 : .938*	1538–1608 *1 : 1.0455*
	1	2	3	4	5	6	7	8	9	10	
I	184–178 *1 : .967*	182–183 *1 : 1.005*	164–137 *1 : .835*	184–180 *1 : .978*	74–97 *1 : 1.31*	111–110 *1 : .991*	187–216 *1 : 1.155*	136–116 *1 : .853*	177–168 *1 : .949*	174–181 *1 : 1.04*	1573–1566 *1 : .996*

Grand Total........10,923–11,246
1 : 1.029

TABLE VII.

Showing Summary of the "High" Strain of the Saratoga Stock. Experiment II.

Generation.											Totals.
A				181 ♂–307 ♀ (*1 : 1.696*)							
B	1 149–163 *1 : 1.09*	2 145–152 *1 : 1.05*	3 147–212 *1 : 1.442*	4 208–194 *1 : .932*	5 201–178 *1 : .885*	6 172–198 *1 : 1.151*	7 143–149 *1 : 1.04*	8 240–240 *1 : 1*	9 22–22 *1 : 1*	10 207–231 *1 : 1.116*	1634–1739 *1 : 1.064*
C	2 72–81 *1 : 1.125*		4 122–109 *1 : .893*		6 163–158 *1 : .969*	8 117–118 *1 : 1.008*	9 106–148 *1 : 1.396*	10 144–136 *1 : .944*			724–750 *1 : 1.036*
D	1 171–192 *1 : 1.123*	2 132–157 *1 : 1.189*	3 119–203 *1 : 1.706*	4 189–324 *1 : 1.714*	5 191–221 *1 : 1.157*	6 175–212 *1 : 1.211*	7 140–222 *1 : 1.586*	8 127–189 *1 : 1.488*	9 97–169 *1 : 1.742*	10 136–149 *1 : 1.096*	1477–2038 *1 : 1.3798*
E	1 107–143 *1 : 1.34*	2 111–180 *1 : 1.62*	3 152–144 *1 : .95*	4 93–109 *1 : 1.17*	5 88–104 *1 : 1.18*	6 207–181 *1 : .874*	7 70–106 *1 : 1.51*	8 168–228 *1 : 1.36*	9 98–99 *1 : 1.01*	10 33–43 *1 : 1.30*	1127–1337 *1 : 1.186*
F	2 258–276 *1 : 1.07*	3 50–37 *1 : .74*	4 118–107 *1 : .907*	5 119–102 *1 : .86*	6 260–221 *1 : .85*	7 81–104 *1 : 1.284*	8 33–28 *1 : .85*	9 163–206 *1 : 1.264*		10 49–56 *1 : 1.14*	1131–1137 *1 : 1.0053*
G	1 33–84 *1 : 2.55*	2 82–111 *1 : 1.35*	3 152–138 *1 : .91*	4 32–41 *1 : 1.28*	5 78–94 *1 : 1.21*	7 42–38 *1 : .88*	8 27–30 *1 : 1.11*	9 89–91 *1 : 1.022*			536–627 *1 : 1.1698*
H	2 74–79 *1 : 1.068*		3 154–151 *1 : .9805*	4 108–129 *1 : 1.19*	5 40–28 *1 : .70*	6 99–89 *1 : .899*		9 37–42 *1 : 1.135*			512–518 *1 : 1.0117*
I	1 63–81 *1 : 1.28*		3 22–15 *1 : .68*		4 60–56 *1 : .93*	6 68–49 *1 : .72*		8 23–18 *1 : .78*			236–219 *1 : .928*

Grand Total........7,377–8,365
1 : 1.134

has made the difference between the two strains, the difference should be most evident in the later generations of the experiment. This is not true, for a moment's calculation will show that the last three generations in each strain do not show as extreme an average ratio in the desired direction as those which preceed tehm. Although the difference between the totals of the two strains may be sufficiently large to be considered significant, it seems more probable that it is a chance variation, especially since the first experiment showed a similar variation in the direction opposite selection.

SELECTION EXPERIMENT III. (Warsaw Stock.)

In order to make the work still more conclusive, selection was attempted upon a third stock. This stock was collected at Warsaw, Indiana, and kept as a stock culture in the laboratory for about six months before the experiment was started.

Here forty-two pairs were mated from among the offspring of a single pair and the extremes of these matings were used as the parents of the high and low strains.[3] There were 11,190 flies examined before the selection began, 5,448 males to 5,742 females, a ratio of 1 : 1.0539. The counts of these matings are given in Table VIII.

TABLE VIII.

Showing Origin of the "High" and "Low" Strains of the Warsaw Stock.

♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
38	43	91	82	125	106	226	220	255	247
66	72	106	101	155	150	110	114	136	143
133	169	135	156	**235**	**238**[2]	80	72	84	77
192	208	41	39	102	105	74	93	94	100
85	75	105	128	48	61	114	129	187	200
147	**187**[1]	178	189	186	186	195	203	89	79
170	192	91	102	127	142	179	199	175	171
105	113	101	104	187	220	158	156		
125	119	106	114	112	138				

Grand Total.............................. 5,448 ♂'s to 5,742 ♀'s

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[1] From this mating originated the "high" strain.

[2] From this mating originated the "low" strain.

[3] It will be seen in Table VIII. that the pair used as the parents of the low strain did not produce the most extreme ratio in that direction. This mistake was due to a mathematical error which was not checked up until it was too late to rectify the experiment.

In the latter part of this experiment there was a diveation from the usual methods. The element of time was sacrificed to be able to make a larger number of matings in each generation. A number of males and females were isolated from each mating, being examined, and held to be mated after all of the counts for the generation were finished. By this method it was necessary to make matings only from the cultures which had given the most desirable ratios but in so doing a considerable amount of time was lost between each generation.

This third attempt to modify, by selection, the sex-ratio did not result in bringing about any very significant difference between the two strains. (Tables IX. and X. give the results of selection upon this stock.) The ratios computed from the totals in each strain showed a slight difference in the desired direction but this was so slight that it might be due to chance. Therefore it seems that no definite conclusions can be drawn from this experiment. In the low strain the totals were 9,673 males to 9,951 females, or a ratio of 1 : 1.0287 and in the high strain 12,327 males to 12,898 females, or a ratio of 1 : 1.0463.

Conclusion.

Since three distinct and unrelated stocks of flies show no significant effect of selection, it seems safe to conclude from these data that Moenkhaus's conclusion concerning the amenability of the sex-ratio in *Drosophila ampelophila* will not hold.

Discussion of Moenkhaus's Work.

Moenkhaus concludes from his work on the effect of selection on sex-ratios in *Drosophila ampelophila* that sex-ratios are "strongly transmissible and amenable to the process of selection." From his paper it is somewhat difficult to tell whether Moenkhaus intended to say that he had developed by selection, strains characterized by high and low female ratios or that by selection, he simply maintained a high and low strain which he found in nature. His results are given in the discussion which follows.

By way of explanation it might be said that Moenkhaus called the strain in which he selected for a relatively higher female ratio the "female" strain, and the one in which he selected in

TABLE IX.

Showing Summary of the "Low" Strain of the Warsaw Stock. Experiment III.

Generation.													Totals.
A	235 ♂s–238 ♀s (1 : 1.013)												
B	2 183–171 1 : .934	3 266–256 1 : .962	4 141–144 1 : 1.02	5 150–155 1 : 1.033	6 112–139 1 : 1.24	7 227–216 1 : .951	9 224–254 1 : 1.13	10 155–125 1 : .806					1458–1460 1 : 1.0013
C	1 222–207 1 : .932	2 234–252 1 : 1.077	3 231–242 1 : 1.048	4 105–112 1 : 1.067	5 211–254 1 : 1.204	6 164–193 1 : 1.177	7 247–246 1 : .996	8 158–160 1 : 1.013	9 96–115 1 : 1.198	10 111–109 1 : .982			1779–1890 1 : 1.062
D	1 163–151 1 : .926	2 226–238 1 : 1.053	3 141–153 1 : 1.085	4 139–125 1 : .899	5 206–227 1 : 1.10	6 204–209 1 : 1.025	7 200–297 1 : 1.49	8 142–177 1 : 1.246	9 193–187 1 : .969	10 188–196 1 : 1.04			1802–1960 1 : 1.087
E	1 51–46 1 : .902	2 96–111 1 : 1.156	3 37–45 1 : 1.216	4 125–119 1 : .952	5 84–89 1 : 1.059	6 56–48 1 : .857	7 101–101 1 : 1	8 73–68 1 : .93					
E	9 88–92 1 : 1.045	10 101–112 1 : 1.109	11 86–78 1 : .907	12 88–86 1 : .977	13 71–94 1 : 1.324	14 63–84 1 : 1.33	15 53–62 1 : 1.169						1173–1235 1 : 1.053
F	1 151–123 1 : .815	3 100–72 1 : .72	4 192–158 1 : .823	5 135–152 1 : 1.126	6 113–96 1 : .849	7 121–110 1 : .909	8 117–129 1 : 1.102	9 41–32 1 : .78	10 97–80 1 : .83	11 99–123 1 : 1.242			
F	12 95–123 1 : 1.295	13 154–153 1 : .99	14 44–33 1 : .75	15 68–96 1 : 1.41	16 209–202 1 : .966	17 59–57 1 : .97	18 169–189 1 : 1.118	19 70–73 1 : 1.043	20 55–52 1 : .945	21 51–43 1 : .843			2140–2096 1 : .979
G	1 64–64 1 : 1	2 103–97 1 : .94	4 51–72 1 : 1.41	7 25–27 1 : 1.08	9 28–38 1 : 1.36	10 39–26 1 : .67	12 59–79 1 : 1.34	13 36–34 1 : .94	15 42–27 1 : .64	16 79–83 1 : 1.05	18 33–39 1 : 1.18	19 34–22 1 : .647	
G	20 19–37 1 : 1.95	21 55–46 1 : .84	22 29–29 1 : 1	23 88–78 1 : .89	24 34–34 1 : 1	25 89–69 1 : .78	26 104–108 1 : 1.04	27 75–79 1 : 1.05	28 65–71 1 : 1.09	30 115–112 1 : .974	31 55–39 1 : .709		1321–1310 1 : .9916

Grand Total........9673–9951
1 : 1.0287

TABLE X.

Showing Summary of the "High" Strain of the Warsaw Stock. Experiment III.

Generation.											Totals.
A						147 ♂s–187 ♀s (*1 : 1.272*)					
B	1 126–115 *1 : .913*	2 195–210 *1 : 1.08*	3 52–42 *1 : .81*	4 53–76 *1 : 1.43*	5 119–127 *1 : 1.07*	6 138–143 *1 : 1.04*	7 191–188 *1 : .98*	8 194–196 *1 : 1.01*	9 206–189 *1 : .92*	10 208–253 *1 : 1.22*	1482–1539 *1 : 1.039*
C	1 76–95 *1 : 1.25*	2 168–202 *1 : 1.20*	3 209–204 *1 : .98*	4 80–85 *1 : 1.06*	5 228–242 *1 : 1.06*	6 59–66 *1 : 1.12*	7 154–170 *1 : 1.10*	8 183–176 *1 : .962*	9 160–179 *1 : 1.12*	10 181–149 *1 : .82*	1498–1568 *1 : 1.047*
D	1 43–43 *1 : 1*	2 69–78 *1 : 1.13*	3 79–90 *1 : 1.14*	4 141–152 *1 : 1.08*	5 149–141 *1 : .946*	6 147–139 *1 : .946*	7 194–190 *1 : .979*	8 195–202 *1 : 1.04*	9 193–181 *1 : .94*	10 146–152 *1 : 1.04*	1356–1368 *1 : 1.009*
E	1 28–39 *1 : 1.39*	2 36–41 *1 : 1.14*	3 74–93 *1 : 1.26*	4 59–70 *1 : 1.19*	5 112–116 *1 : 1.04*	6 123–128 *1 : 1.04*	7 107–121 *1 : 1.13*	8 94–91 *1 : .97*			
E	9 92–105 *1 : 1.14*	10 52–62 *1 : 1.19*	11 84–82 *1 : .976*	12 67–121 *1 : 1.806*	13 105–126 *1 : 1.2*						1033–1195 *1 : 1.157*
F	1 145–146 *1 : 1.007*	2 214–245 *1 : 1.15*	3 249–246 *1 : .988*	4 28–65 *1 : 2.32*	5 168–179 *1 : 1.07*	6 169–162 *1 : .96*	7 154–162 *1 : 1.05*	8 77–63 *1 : .82*	9 133–146 *1 : 1.098*	10 79–74 *1 : .94*	

TABLE X.—*Continued.*

F	11	12	13	14	15	16	17	18	19	20		
	100–102	214–228	195–226	182–199	177–149	208–214	194–207	121–135	148–157	93–86		
	1 : 1.02	*1 : 1.07*	*1 : 1.16*	*1 : 1.09*	*1 : .842*	*1 : 1.03*	*1 : 1.07*	*1 : 1.12*	*1 : 1.06*	*1 : .93*		
F	21	22	23	24	25	26	27	28				
	132–127	75–98	115–149	114–122	192–206	113–130	118–136	173–156				4080–4315
	1 : .96	*1 : 1.31*	*1 : 1.3*	*1 : 1.07*	*1 : 1.07*	*1 : 1.15*	*1 : 1.15*	*1 : .902*				*1 : 1.0576*
G	1	2	3	4	5	6	7	9	10	11	12	
	57–69	58–56	85–91	49–56	33–27	84–72	26–46	44–49	90–94	112–122	92–83	
	1 : 1.21	*1 : .97*	*1 : 1.07*	*1 : 1.14*	*1 : .82*	*1 : .86*	*1 : 1.77*	*1 : 1.11*	*1 : 1.04*	*1 : 1.09*	*1 : .90*	
G	13	14	15	16	17	18	19	20	21	22	23	
	65–67	46–51	128–109	73–75	53–49	73–86	41–44	62–49	34–32	68–76	63–98	
	1 : 1.03	*1 : 1.11*	*1 : .85*	*1 : 1.03*	*1 : .93*	*1 : 1.18*	*1 : 1.08*	*1 : .79*	*1 : .94*	*1 : 1.12*	*1 : 1.56*	
G	24	25	26	27	28	29	30	31	32	33	34	
	36–27	39–60	80–69	53–33	52–62	134–108	42–35	50–48	49–39	103–113	71–87	
	1 : .75	*1 : 1.54*	*1 : .86*	*1 : .62*	*1 : 1.19*	*1 : .81*	*1 : .83*	*1 : .96*	*1 : .796*	*1 : 1.097*	*1 : 1.23*	
G	35	36	37	38	39	40	41	42	43	44	45	
	113–109	58–57	84–94	32–27	29–32	60–50	61–64	27–36	67–70	152–126	50–66	2878–2913
	1 : .97	*1 : .98*	*1 : 1.12*	*1 : .84*	*1 : 1.10*	*1 : .83*	*1 : 1.05*	*1 : 1.33*	*1 : 1.05*	*1 : .829*	*1 : 1.32*	*1 : 1.0122*

Grand Total........12,327–12,898
1 : 1.0463

the opposite direction the "male" strain. This terminology will be used throughout this discussion. As the starting point of his female strain, he selected from nature a pair of flies which produced 52 males and 135 females and in his male strain he started with a pair which produced 84 males and 75 females.

Considering first the effect of selection upon his female strain we find here no conclusive evidence of any progressive change in the direction of the selection. (His data for this strain are given in Table XI.) To be sure, there is a slight increase in the relative number of females in his second and third generations over his first generation, but this fluctuation could easily be attributed to the small number of individuals examined. The greatest difficulty in assuming the effectiveness of selection here, is the fact that his last two generations are the ones in which he obtained the lowest relative number of females. Moenkhaus explains these low ratios of the last two generations in that he possibly made a poor selection in the preceding generation, but if we consider selection as having a cumulative effect (as Moenkhaus seems to consider it) it is difficult to see how one could lose by one poor selection all that he had accomplished in the previous selections. It must be admitted that by using as parents of each succeeding generation, pairs which threw the most extreme ratios in favor of the females, Moenkhaus was able to maintain a strain which on an average gave a relatively high number of females. But this was probably not due to any cumulative effect of selection but to the isolation of a peculiar type of female which will be discussed more fully later.

As to the male strain (Table XII)., Moenkhaus admits that the effect of his selection here has been slight and after examining his data carefully it is difficult to see how it could be assumed that there has been even a slight effect of selection. He started with a ratio (84 males to 75 females) which was only slightly different from normal and this slight difference was not transmitted to the first generation nor any succeeding one. So it seems that he had here a normal strain which would have given the same ratio regardless of the direction of the selection.

The female strain is the unusual one and is in need of an explanation. It throws some very exceptional ratios in favor of

TABLE XI.

History of Moenkhaus's "Female" Strain.

206 (*52* ♂s : *135* ♀s)

Generation.												Totals.
	1	2	3	4	5	6	7	8	9	10	11	
1	21–37	42–68	71–164	76–71	108–125	51–80	43–67	51–46	53–103	8–56	55–102	578–969 *1 : 1.67*
						7	8	9	10			
2						21–49	41–94	55–70	98–178			215–391 *1 : 1.82*
				3	4	5	7					
3				15–37	18–54	25–37	35–71					93–201 *1 : 2.17*
	1	2	3	5	7	8	10					
4	26–47	39–56	33–52	64–67	53–93	85–162	54–106					354–483 *1 : 1.36*
					4	5	6	7	8			
5					121–139	32–32	93–101	40–114	86–132			372–518 *1 : 1.39*
	2	3	5	6	7	10	13					
5	51–44	67–53	41–47	74–78	120–130	102–136	41–47					496–535 *1 : 1.07*

TABLE XII.

History of Moenkhaus's "Male" Strain.

207 (*84* ♂s : *75* ♀s)

Generation	1	2	3	4	5	6	7	8	9	10	11	Totals.
1		79–68			99–121	71–88	67–73	60–57		72–71	86–100	536–579 *1 : 1.08*
2			44–50		133–140		25–21		18–21			220–223 *1 : 1.00*
3	71–98	115–101	76–84	51–73	42–37	45–48	36–39	61–52	84–108			581–640 *1 : 1.10*
4	10–14				41–41	38–38	45–47					142–147 *1 : 1.04*
5	40–32		45–144		46–35	26–26	146–144	72–98	64–74		79–84	518–607 *1 : 1.17*
6	30–50	94–116		58–67	32–51		55–75	85–92	64–104	43–99		561–654 *1 : 1.42*
6	118–109	99–107	164–198	141–157	61–85	56–63	24–27	116–109	42–40	73–67	50–35	944–997 *1 : 1.05*

the females and these exceptional individual ratios caused the relative number of females in the female strain to be higher than normal. These unusual ratios can be readily explained by assuming that the female which Moenkhaus selected as the mother of his female strain carried a recessive sex-linked lethal factor and it will be pointed out later that all of Moenkhaus's data substantiate, very precisely, this assumption. The existence of such factors has been conclusively demonstrated by the recent work of Morgan and his students. A recessive lethal factor is any factor that brings about the death of the individual in which it occurs, provided its effect is not counteracted by the action of its normal allelomorph. Then if a lethal is sex-linked, all males which get it will die for they cannot carry its normal allelomorph since they possess but one X-chromosome. Since all males receiving the lethal factor die, this factor is never transmitted by the male and as a consequence the female can never be homozygous for it. Therefore the lethal factor has no effect on the female but she, by transmitting it to half of her sons, causes their death. Since half of the males die, a 2:1 ratio will result. A female carrying a lethal will transmit it to half of her daughters and they will always be heterozygous for it since they cannot receive it from their father.

Then the female which Moenkhaus used as the mother of his female line was probably a lethal female. It should be said that Moenkhaus is not to be criticised for not considering lethals in his paper, for lethals were not known till two years after his work was published. The original female gave a ratio which was an approximate 2:1 ratio. As mothers of the succeeding generations, Moenkhaus probably selected lethal bearing females (excluding female 5 of generation 4). These females should transmit the lethal factor to half their daughters and this expectation is realized, for from the 31 matings made in the female strain (excluding the offspring of female 5 of generation 4), fifteen 2:1 ratios resulted. Those considered 2:1 ratios are matings 3, 9 and 11 of generation 1; 7, 8 and 10 of generation 2; 3, 4 and 7 of generation 3; 1, 7, 8, and 10 of generation 4; and 7 and 8 of generation 5. So the number of 2:1 ratios obtained would justify the assumption of the presence of a lethal factor.

Another indication of the presence of a lethal factor is the fact that in generation 4, when he used female 5 which gave a normal ratio, as mother of the next generation, all of her daughters also threw normal ratios. This female did not carry the lethal factor and none of her offspring showed abnormal ratios.

A third indication of a lethal factor is the fact that Moenkhaus found in crossing his male and female strains that the female is almost wholly responsible for the transmission of the sex-ratio. For he found if females from a strain possessing a high female ratio be mated with males from a strain possessing a low female ratio or vice versa, the offspring will show a sex-ratio which is wholly or very near that of the strain from which the females were taken. If his female strain carried a lethal factor and if his male strain was a normal strain, as it appears to be, the above result would be the expected one. For, if a lethal bearing female is mated to a normal male, she will transmit the lethal factor to half her daughters but a male from the lethal strain cannot transmit the lethal factor because all of the lethal bearing males die. Therefore the end result would be as Moenkhaus found, that the offspring will show sex-ratios like that of the strains from which the females came.

Of course, the only sure way of testing for a sex-linked lethal factor is to cross the suspected female to a male carrying another sex-linked factor and the resulting F_2 will be characterized by a deficiency in the class of normal males. So it is not possible to determine beyond doubt whether the unusual ratios in Moenkhaus's female strain were due to a lethal factor. But since several lethals have already been found and all of his data substantiate this assumption, it seems that this explanation is probably the correct one.

DISCUSSION.

The writer's three attempts to modify by selection, the sex-ratio in the fruit fly have resulted in no clear-cut evidence that it was modifiable. In each experiment, the work covered more generations and many more individuals than the work of Moenkhaus. Since Moenkhaus's results could not be obtained in any one of the three experiments, it leads one to question Moenkhaus's conclusions or, at least, to question the general application of his findings. The fact that the difference which Moenkhaus found between his two strains can be readily explained to be due to a cause entirely independent of the cumulative effect of selection, makes his work support, rather than oppose the findings of the writer. It must be admitted that negative results in secletion work can never be conclusive, for the criticism may always be offered that selection over a greater period might have yielded different results. But, nevertheless, we feel justified in concluding from the data here presented that the sex-ratio in *Drosophila ampelophila* is not readily, if at all, modifiable by selection.

LETHALS.

Since the offspring of over 700 pairs of flies were examined for sex in these experiments, and since extreme ratios were sought, it would not be surprising to find a sex-linked lethal mutation. In fact, there is fairly good evidence that one such mutation occurred. This was mating number of 6 generation G of the low strain of the Bloomington stock (Table IV.). This gave an approximate 2: 1 ratio (84 males to 180 females). Four matings were made from among the offspring of this mating and three out of the four gave 2 to 1 ratios while the fourth was doubtful. If a sex-linked lethal mutation had occurred, half of the four should have given 2 to 1 ratios and the other half normal. Since so few matings were made, it is possible that all the females chosen were lethal bearers. The stock was lost by accident at this time and further tests were not possible. But since the unusual ratio was transmitted it is probable that a mutation occurred here. In the high series of the Saratoga stock, there were several ratios which approximate a 2 to 1 ratio but none of the flies showing it transmitted this tendency to their offspring. Also in the high strain of the Warsaw stock, generation E mating 12 gave a 2 to 1 ratio. A large number of matings were made among the offspring of this pair but this unusual ratio was not transmitted to any of the progeny.

In all of the matings examined there were comparatively few ratios found which were two to one or more.[1] These extreme ratios were more frequently in favor of the females and in only one case was there any evidence that a sex-linked lethal mutation had occurred.

---

[1] The unusual ratios more frequently occurred where the counts were very small. These cases were considered to have been due to some unfavorable environmental condition.

In conclusion, I wish to express my thanks to Professor Fernandus Payne for the suggestion of this problem and also for many helpful suggestions and criticism while the work was in progress. I am also greatly indebted to my wife, Elmira Shierling Warren, for help, both in the carrying out of the experiment and in the preparation of the manuscript.

SUMMARY.

1. The sex-ratio in *Drosophila ampelophila* is 100 females to 95 males.

2. The age of the parent has no effect upon the sex-ratio of its offspring.

3. The difference which Moenkhaus found between his two strains, and which he attributed to selection, was probably due to the fact that his male strain was a normal one and his female strain was a lethal bearing one.

4. The sex-ratio in *Drosophila ampelophila* is not readily, if at all, modifiable by selection.

5. There was probably one sex-linked lethal mutation in the writer's selection stocks.

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EFFECTS OF AGING UPON GERM CELLS AND UPON EARLY DEVELOPMENT.

Part II., Changes in Moderately Aged Eggs and Sperm.

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In a previous publication (Goldfarb, '17), the writer determined the variation of freshly liberated sperm and eggs from freshly collected sea urchins of three different species. Two of these, *Toxopneustes* and *Hipponoë*, inhabit the shallow tropical waters of the Dry Tortugas, and the third, *Arbacia*, is common in the deeper colder waters near Woods Hole, Mass. In each species preliminary experiments had been made to ascertain the optimum conditions of development, such as volume and surface and filtration of sea water, concentration of eggs and sperm, etc., and such optimum conditions were used in all subsequent experimentation.

It was shown that even under these constant and optimum conditions, the freshly liberated eggs of different females varied in respect to (1) size, (2) jelly layer, (3) membrane formation, and (4) cleavage; that there was a surprisingly large variation, with respect to each of these four classes, in the eggs of different females; that the sperm of different males also varied, but to a much less degree. And finally the range of variation was determined for each species.

It was suggested that these variations served as convenient and accurate indices of the physiologic condition or vitality of the freshly removed eggs or sperm of different individuals.

In the present paper the same technique, the same three species of sea urchin, the same nomenclature and the same precautions were taken. It is proposed to determine first the physiologic condition of the germ cells, at the time of liberation, from given individuals, and then to determine the nature and the extent of the changes in the eggs and sperm as they become increasingly "overripe," "aged" or" stale."[1] In the next study, Part III., it is proposed to study other changes that appear in very late stages of overripening or ageing of the germ cells. I reserve for Part III. a discussion of the entire problem, and the bibliography.

The changes that were observed in moderately aging eggs, and those which will be discussed in detail are: (1) size, (2) jelly layer, (3) membrane formation and (4) cleavage.

I wish to acknowledge my indebtedness to Dr. A. G. Mayer, Director of the Marine Biological Laboratory of the Carnegie Institution of Washington, and to Prof. F. R. Lillie, Director of the Marine Biological Laboratory at Woods Hole, Mass., for the opportunity of working on this problem at their respective laboratories, and for the many facilities offered in connection with this work.

Effect of Age Upon Size of Eggs.

Hipponoë and Arbacia.

The diameters of freshly removed unfertilized eggs of each freshly collected female, were measured with an ocular micrometer (no. 3 eye piece and 1/6 objective) and the usual precautions concerning pressure, focusing, sampling, etc., taken. At different intervals (ages) other samples of the eggs of the same female were measured. The data are brought together in Table I.

I have shown (Goldfarb, '17) that the normal size of freshly liberated eggs of *Hipponoë* varied from 18½ to 20 ocular units in diameter, with a mode of 19½ units. In the present series

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[1] By age is meant the time since removal or extrusion of ripe germ cells.

TABLE I.

SHOWS CHANGE IN SIZE AND LOSS OF JELLY LAYER WITH AGE. SIZE IS GIVEN IN OCULAR MICROMETER UNITS OF THE DIAMETER OF THE EGGS AT DIFFERENT AGES. *Hipponoë.*

No.	Date.	Age of Eggs, Hrs.	Diameter of Eggs in Ocular Micrometer Units.									Per Cent. of Eggs with Jelly Layer.	Per Cent. Loss of Jelly per Hour.
			21.0.	20.5.	20.0.	19.5.	19.0.	18.5.	18.0.	17.5.	17.0.		
			Number of Eggs.										
1	6/15	5				*	*						
		22	*	*	*								
2	6/23	5				1	8	1				97	
		29				4	7	1				78	0.7
3	6/28	½				1	7	4				100	
		18½				8	18	3				100	0.0
4	6/28	½			2	5	6	2				100	
		18½			3	8	5	0				94	0.3
5	6/25a	2			2	7	1					100	
		5		2	8	3	1					91	
		24		7	1	3	2					40	2.7
6	6/27a	3			1	12	3					96	
		9			12	5	1					96	
		27		1	9	2	0					79	0.7
		33		0	13	3	0						
7	6/26a	0		1	3	6	3					86	
		18		3	3	6	0					77	
		24				9	5	1				62	1.0
		40						1	9	6	5	0	
		47							1	9	2	0	
8	6/26b	0			8	3						95	
		18	2	4	8	4						95	
		24			7	3						72	0.9
9	6/26c	0				0	4	8				90	
		18				0	15	2				60	
		24				3	8	3				6	3.5
		40				3	12	1				2	
		47				1	6	6				1	
10	6/25b	2				1	9	1				63	
		5				1	10	1				1	
		24					5	7	4	1		0	2.8
11	6/27b	3	2	4	3	6	0	0				80	
		9			1	4	9	1				10	
		27			1	9	2	0				1	3.3
		33			1	2	3	0	2	2	1	0	
12	6/26c	0			5	10						99	
		18		2	12	2						98	
		24		0	12	1	1					98	0.4
		40		1	11	1	1					62	
		47		1	12	1	0					17	
Variation in freshly liberated eggs of different females...			2	6	34	72	54	16	0	0	0	90	

the same variations occurred in the freshly liberated germ cells. As the eggs aged, there was a clear, considerable and progressive change in size. The observations may best be considered in three groups.

Female 1, is an example of the first group. When her eggs were 5 hours old, *i. e.*, 5 hours after liberation, their diameters were 19 and 19.5 units. When her eggs were 22 hours old, they measured 20, 20.5 and 21 units, *a clear and sharp increase in minimum, mode and maximum diameters, with age.*

In female 2, observations were made when the eggs were 5 and 29 hours old. In this female there was also an enlargement but very small. In females 3 and 4, observations were made at ½ and 18 hours respectively. In each female the eggs enlarged with age. In female 5, three observations were made, viz., 2, 5 and 24 hours, with a progressive enlargement at each successive age. In female 6, observations were made at four intervals, 3, 9, 27 and 33 hours. As in female 5, there was at first a rapid enlargement of the eggs, then a slow increase, and, in very late aging, a rapid increase again. The average diameters at each of the four intervals was 19.44, 19.80, 19.77 and 19.96.

In all the eggs of this first group, there was a cumulative increase in volume, with age, for the first 33 hours.

The second group includes such females whose eggs were observed over a longer period than 33 hours. In this group as in the first, the eggs also enlarged at first, with age, but after a longer interval there occurred a secondary diminution in size, and in very old eggs became even smaller than the norm.

For example, the eggs of female 7 were measured at 0, 18, 24, 40 and 47 hours. When 18 hours old the eggs were slightly larger than at the first observation. At 24 hours, the eggs were clearly smaller, and at 40 hours smaller still. At 47 hours they were much below their original size and below the norm for the species. The maximum size had diminished from 20.5 to 18, the mode from 19.5 to 17.5, the minimum from 19 to 17.

In female 8, there was the same initial increase and subsequent diminution towards the norm, when 24 hours old. In female 9, observations were made at 0, 18, 24, 40 and 47 hours. There occurred the usual enlargement until the 40th hour, after which there was a definite diminution in volume.

In a third group, of a few females only, the eggs though freshly removed, were in poor physiologic condition. In this group *the initial enlargement did not take place.* In these eggs *there occurred a direct diminution with age.* In this group belong females 10 and 11, and probably female 7.

It should be recalled that the physiologic condition of any set of eggs was not determined merely by egg size, but by the results of several tests, such as the jelly layer test, the membrane test as well as the cleavage test, etc. By all of these tests, it was clearly and definitely shown *that eggs in good physiologic condition at the time of liberation, increased steadily with age, within the limits shown in Table I., and subsequently diminished in size by a process of fragmentation described in Part III. Eggs in physiologically poor condition at the time of liberation behaved like half-aged eggs, that is, they either enlarged but little, or did not enlarge at all, and subsequently diminished to and below the norm of the species.*[1]

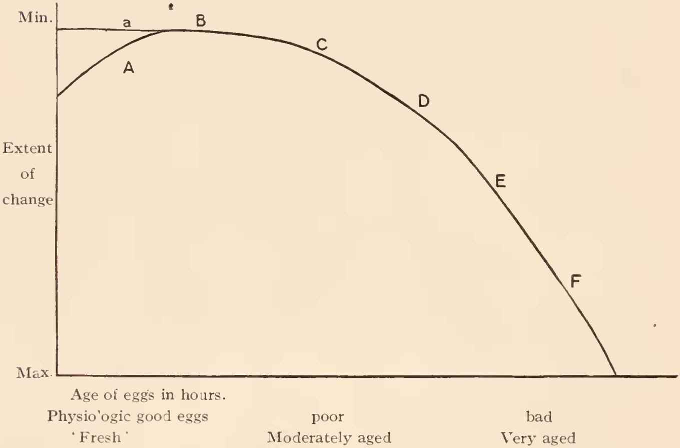

FIG. 1. Extent of morphologic and physiologic change in aging or deteriorating eggs. *A′, B, C, D, E, F,* represents the change in volume, rate of membrane formation, rate of cleavage, total cleavage (*Hipponoë*). *A, B, C, D, E, F,* represents the change in width of fertilization membrane, loss of jelly, total cleavage, etc. *A′, B* is period of superripening; *B, C, D,* the period of overripening or deterioration; *D, E, F,* the period of extreme deterioration or dying of the eggs.

Figure 1 represents graphically and schematically the change in volume with age. Physiologically good eggs show an initial rapid increment, region *A* of the curve; then a progressively slower increment, region *B;* thirdly a progressive *diminution*, region *C*, towards *D* or even below the norm of the species to *E*. Eggs in moderately poor physiologic condition at liberation have already undergone physiologic deterioration, corresponding with part *A* within the body of the mother. Upon liberation they show only parts *B*, *C*, *D*, etc., of the curve. Eggs in very poor condition upon liberation have undergone changes represented by parts *A* and *B*, within the body of the mother, and upon liberation show only parts *C* and *D*, etc.

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[1] So constant is the relation between volume and physiologic condition, tha one can predict the one from the other, with remarkable exactness.

The significance of this increment with age lies in the changed permeability of the cortical layer which is denoted by increase in volume. This progressive increase in permeability permits increasing volumes of sea water to penetrate the egg and thus enlarge it. This process continues until partial cytolysis (fragmentation) reduces the size once again towards the norm, or until complete cytolysis destroyed the eggs altogether.

The change in size with age really measures the change in permeability of the cortical layer.

Similar observations were made upon *Arbacia* eggs, and the changes are so much in agreement with those of *Hipponoë* that it seems unnecessary to describe them here. There is the same initial increment followed by the secondary diminution with age. And the rate of change depended upon the physiologic condition of the eggs at the time of liberation and upon temperature. The two species differ in the markedly slower rate of change of *Arbacia*, due in largest part to the lower temperature of the sea water.

Effect of Age Upon the Jelly Layer.

Hipponoë and Arbacia.

The jelly layer of the same sea-urchin eggs were made clearly visible by adding Chinese black to the sea water, as suggested by F. R. Lillie. Samples of 150 to 200 consecutive freshly removed (and unfertilized) eggs of each female were examined, avoiding mechanical or chemical removal of the jelly, and the presence or absence of the jelly layer recorded. At successive intervals other samples of the same female were examined, and the results reduced to percentages as recorded in Table I.

I have shown (Goldfarb, '17) that the freshly liberated eggs of different females vary considerably in the per cent. possessing the jelly layer. In *Hipponoë* the per cents vary from 100 to 63 per cent., in *Arbacia* from 100 to 59 per cent. These differences, I suggested, indicated corresponding differences in physiologic condition of the eggs at the time of liberation.

Whatever the condition of the eggs at the time of liberation, with increasing age there was a continuous decrease in the number of eggs with the jelly layer present.

The data may best be considered in two groups, according to the physiologic condition of the eggs at the time of the first observation.

In the first group are those females whose eggs were in good physiologic condition. This was indicated by the high per cent. (*i. e.*, above 95 per cent. with jelly layers) at the first observation, as well as by other tests. In this group are placed females 2, 3, 4, 5, 6, 8 and 12, and the corresponding percents of jelly layers was 97, 100, 100, 100, 96, 95 and 99 respectively.

In the second group are placed those females whose eggs were in poor physiologic condition. In this group are included females 7, 9, 10 and 11, whose corresponding jelly counts were, 86, 90, 63 and 80 per cent., respectively.

In both groups the surrounding jelly layer progressively disappeared with age, as seen in Table I. But the rate of loss was different. An example of the first group is female 8. When 0 hours old, 95 per cent. possessed the jelly layer, when 18 hours old 95 per cent., and when 24 hours old only 72 per cent. retained the layer. Female 11 is an example of the second group in which the loss was decidedly greater. The eggs when 3 hours old showed 80 per cent. with jelly layers, when 9 hours old but 10 per cent., when 27 hours old only 1 per cent., and when 33 hours old none of the eggs possessed the jelly envelope.

The two groups differ then, not only in original per cent. with jelly layers, but also in the rate of loss of this layer. In the first, containing physiologically good eggs, the loss of the jelly layer per hour, for the indicated intervals for the different females, was 0.7, 0.0, *0.3, 2.7, 0.7, 0.9, 0.4 per cent., with an average of 0.81 per cent. loss per hour. In the second group with physiologically poor eggs the loss was, 1.0, 3.5, 2.8, 3.3 per cent. per hour with an average of 2.65 per cent. or over three times as rapid.* I cannot account for the only apparent exception, namely female 5, whose rate of loss was 2.7 per cent.

Fig. 1 represents the loss of jelly with age. With physiologically good eggs, the numbers with jelly is a maximum or nearly so, and the rate of loss with age is small, part *a* and *B*. As the eggs become physiologically poor, and in eggs which were in poor physiologic condition at the time of liberation, the numbers with jelly is much less, and the rate of loss with age is more rapid, parts *C*, *D*, etc. It should be noted in passing that parts *B*, *C*, *D* and *E*, are parallel with the corresponding parts of the curve showing change in the volume of eggs with age.

The observations upon *Arbacia* eggs are in entire accord with those of *Hipponoë*. They differ only in the slower rate of loss. One example may illustrate this slower rate. The eggs when freshly removed showed 90 per cent. with their jelly layers. When 41 hours old, 50 per cent. possessed the layer, when 46 hours, 19 per cent., when 70 hours, 1 per cent., and when 72 hours none of the eggs possessed the jelly layer. *In both species of eggs the loss of jelly was a function of age, and physiologic condition at time of the first observation.* If the loss of jelly is known, one can predict (provided proper precautions are taken) the physiologic condition of the eggs and vice versa, from the physiologic condition one may determine the loss of jelly.

Loeb, Harvey and F. R. Lillie in particular, as well as others, have recorded the loss of jelly in old eggs. My observations show not only a loss with age, which is different for different species, but I have determined the rate of loss with age. More than this, I have shown that the rate of loss depends not only upon the species and temperature, but upon the condition of the eggs at liberation, and finally that loss of jelly serves as another symptom of the physiologic condition or vitality of the eggs at any time, and that this vitality diminished at a known rate with age.

Effect of Age upon Membrane Formation.

A third sympton of aging and physiologic deterioration is the change in fertilization membrane. A number of investigators have observed that old eggs do not form fertilization membranes (Hertwig, R. and O., '86, Loeb, '03, '15, Harvey, '10, '14, F. R. Lillie, '14, etc.). Harvey ascertained the exact age in *Arbacia* beyond which, namely 52 hours, no membranes were formed. Just, '15, observed that the jelly layer, which follows fertilization in *Nereis* was retarded with aging.

Before considering in detail the changes in the fertilization membrane, with age, and the causes of such changes, it should be recalled that freshly liberated eggs of freshly collected females, when fertilized under constant and optimum conditions, varied considerably in the rate of membrane formation. Such freshly liberated eggs may be placed into three groups according to their physiologic condition, namely (1) those that form membranes within 2 minutes after fertilization, which are in good physiologic condition. (2) Those that form membranes in 3 to 7 minutes, and which are in moderately poor condition, and (3) those that do not form membranes, which are in poor physiologic condition. It should also be recalled that the rate of membrane formation is further complicated by the differential effect of eggs and sperm. I will mention but two of the experiments, which are typical, to illustrate this differential effect. In experiment 1, Table II., *the eggs of one female were fertilized by the sperm of 5 different males*. All the germ cells were 43 minutes old. Four of the males induced membrane formation in 1½ minutes, the fifth in 2 minutes. Even greater uniformity occurred when the germ cells were 1 hour old, for *the eggs of all 5 samples of the same female formed their membranes at the same time, namely 1 minute*. When the germ cells were 1½ and 2¼ hours old, the eggs again reacted in the same manner for none formed membranes.

In contrast to this uniformity of behavior, when the eggs of one female were fertilized by different males, are those experiments in which the eggs of different females were fertilized by the one male. For example, in experiment 2, the eggs of 3 females were fertilized by the one male, when the germ cells were 20 minutes old. Fertilization membranes first appeared in the three groups of eggs as follows, $3\frac{1}{2}$, $1\frac{1}{2}$ and $1\frac{3}{4}$ minutes respectively. When the germ cells were 80 minutes old, they appeared in $2\frac{2}{3}$, $1\frac{2}{3}$, $1\frac{5}{6}$ minutes; when 140 minutes old, $1\frac{2}{3}$, $1\frac{1}{2}$ and 1 minute; when 280 minutes, $\frac{2}{3}$, $1\frac{1}{4}$ and 1 minute.

In the other experiments similar results were obtained, i. e., uniform or nearly uniform rate of membane formation, when the eggs of one female were fertilized by different sperm, and frequently,

TABLE II.*a.*

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION WITH AGE, OF EGGS OF DIFFERENT FEMALES. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Minutes Required Before first Membranes Appeared.						Average No. Minutes.
			Male 1.	2.	3.	4.	5.	6.	
1	7/3	43	2	$1\frac{1}{2}$	$1\frac{1}{2}$	$1\frac{1}{2}$	$1\frac{1}{2}$		1.6
		59	1	1	1	1	1		1.0
		89	N	N	N	N	N		N
		140	N	N	N	N	N		N
			Female 1.	2.	3.	4.	5.		
2	7/14	20	$3\frac{1}{2}$	$1\frac{1}{2}$	$1\frac{3}{4}$				2.25
		80	$2\frac{2}{3}$	$1\frac{2}{3}$	$1\frac{5}{6}$				2.05
		140	$1\frac{2}{3}$	$1\frac{1}{2}$	1				1.39
		210	$1\frac{1}{4}$	$1\frac{1}{4}$	$1\frac{1}{4}$				1.25
		280	$\frac{2}{3}$	$1\frac{1}{4}$	1				0.97
		370–600	N	N	N				N
3	7/21	120	$1\frac{1}{2}$	1					1.25
		323	$1\frac{1}{3}$	1					1.15
		450	N	N					N
4	7/16	160	N	N	$2\frac{1}{2}$	$2\frac{1}{2}$	0	$2\frac{1}{2}$	2.5
		300	N	N	N	N	N	$1\frac{1}{2}$	0.5
		500–600	N	N	N	N	N	N	N
5	7/7	75	2	3	$2\frac{1}{2}$	$3\frac{1}{2}$			2.75
		84	1	2	$1\frac{1}{2}$	2			1.62
		180	2	2	$4\frac{1}{2}$	6			3.62
6	7/12	17	$1\frac{2}{3}$	1	1				1.22
		42	$1\frac{1}{2}$	1	1				1.16
		99	$\frac{1}{2}$	$\frac{3}{4}$	$\frac{3}{4}$				0.66
		161	$\frac{1}{3}$	1	$\frac{2}{3}$				0.66
		214	$\frac{1}{5}$	$\frac{2}{3}$	$\frac{3}{4}$				0.53
		294	$2\frac{1}{2}$	$\frac{5}{6}$	N				1.11
		360	$5\frac{1}{2}$	N	N				1.83
		420	N	N	N				N
7	7/19	130	3	3	2	$1\frac{1}{2}$	$1\frac{1}{2}$	$1\frac{1}{2}$	2.08
		240	N	2	2	$1\frac{1}{3}$	$1\frac{1}{3}$	$1\frac{1}{2}$	1.36
		350	N	2	2	2	2	2	1.83
		470	N	$3\frac{1}{2}$	4	N	$2\frac{1}{2}$	$2\frac{1}{2}$	2.08

N = no membrane in 10 minutes.

wide differences when the eggs of different females were fertilized by the one male. There was a far greater variation among the eggs of different females than among the sperm of different males, or, putting the matter in a slightly different way, the extent of variation in development is conditioned much more by the eggs than by the sperm.

With these facts in mind, we may proceed with the examination of the effect of age upon the membrane formation in physiologically "good," "moderately poor" and "poor" eggs. The data are brought together in Tables II.*a* and II.*b*.

TABLE II.*b*.

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION OF AGEING EGGS AND SPERM.

Arbacia.

No.	Date.	Age of Germ Cells in Hours.		Minutes Required Before Membranes Appeared.						Average No. Minutes.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.	
1	8/12	4	4	2	1½	3	2	2		2.1
		23	23	3	7	N	N	N		5.0
		28–48	28–48	N	N	N	N	N		N
2	8/14	4	4	2	2	2	2	2	2	2.0
		24	1	N	N	3½	2	N	N	2.7
		31	8	N	N	N	N	N	N	N
		49	1¼	N	N	N	N	N	N	N
		5	5	1½	1½	½	½	½		
3	8/16	1½	1½	1½	1½	1½	1½	1½	1½	1.5
		18½	18½	3½	N	3½	3½	3½	N	3.5
		23	23	N	N	N	N	N	N	N
		24	4	2½	N	2½	N	6	N	3.6
		42	1	N	N	N	N	N	N	N
		48–75.	⅒–¾	N	N	N	N	N	N	N
4	8/18	1	23	7	3	3	3			
5	8/19	1	45½	2	N	2½				
6	8/20	5	73	2½	4	4½	5			
		10	95	2½	N	N	N			

N = no membranes in the first 10 minutes.

In the first group, *containing physiologically good eggs, the rate of membrane formation was accelerated with age.* For example in experiment 2, the eggs of the 3 females were fertilized by one male when the germ cells were 20, 80 140, 210, 280, 370, 440 and 550 minutes old. The eggs of female number 1, for instance, formed their membranes at the above ages in 3½, 2⅔, 1⅔, 1¼ and ⅔ minutes, and none after 370 minutes. The eggs of female number 3 formed their membranes in 1¾, 1⅚, 1, 1¼ and 1 minute and none after 370 minutes. The average for all 3 females at the give ages was 2.25, 2.05, 1.39, 1.25, 0.97 minutes, and none between 370 and 600 minutes when the experiment terminated. A corresponding acceleration in the rate of membrane formation, with age, occurred in all the other experiments with physiologically good eggs.

In the second group of physiologically poor eggs, there was also an acceleration in rate of membrane formation with age, but subsequent to this, there was a period of retardation, and ultimate inability to form membranes.

For example in Experiment 5, the eggs of 4 females were fertilized by the same male when the germ cells were 75, 84 and 180 minutes old. The eggs of female no. 1 formed their membranes at these ages in 2, 1 and 2 minutes respectively, in female 3, in 2½, 1½ and 4½ minutes, in female 4 in 3½, 2 and 6 minutes respectively. Female 2 alone did not show any retardation. The average for all 4 females was *2.75, 1.62 and 3.62 minutes respectively.*

In experiment 6, of the three females whose eggs showed an initial acceleration, only one showed a secondary retardation. The observations in this experiment were made at more intermediate ages namely, 17, 42, 99, 161, 214, 294, 360, 420 and 430 minutes respectively. The eggs of female no. 1 formed membranes in 1 ⅔, 1½, ½, ⅓, ⅕ minutes and formed no more membranes after 420 minutes. The average rate for all three females for the corresponding ages, was *1.22, 1.16, 0.66, 0.53, 1.11 and 1.83 minutes* respectively, and none after 420 minutes.

In experiment 7, 5 out of 6 females showed the initial acceleration with age, and the secondary retardation. The average for the 6 females for the following ages 130, 240, 350 and 470 minutes, was *2.08, 1.36, 1.83 and 2.08 minutes respectively.*

The results may be graphically represented as in Fig. 1. The ordinates represent the inverse time or rate of membrane formation. The abcissas represent age of germ cells. With physiologically good eggs, the time and rate of membrane formation decreases with age, parts *A* and *B* of the curve. Beyond certain limits when the eggs are in poor physiologic condition, the time and rate is correspondingly increased, parts *C*, *D*, *E*, etc. In eggs, physiologically poor at the time of liberation, there is either a short period of acceleration, part *B*, followed by a long period of retardation, parts *C*, *D*, *E*, etc. In eggs in still poorer condition, the retardation takes place from the very beginning of the experiment, parts *C*, *D*, *E*, etc. Hence the better the condition of the eggs the longer the membrane-forming period and the longer the period of acceleration. The poorer the condition the shorter the periods.

I have chosen the reciprocal curve, for in this form it becomes quite clear that it is identical with Fig. 1, showing increase in size of eggs, and decreased per cent. of jelly layers, with age. I will discuss this correlation later.

There is one other matter that deserves brief mention. It was observed repeatedly that physiologically good eggs form membranes a considerable distance from the egg surface. With increasing age and with change in rate of membrane formation, the membranes are formed closer and closer to the egg, until they could barely be distinguished from the plasma membrane, and finally did not appear at all. With this reduction in width of the perivitelline space there appeared to be a greater tendency toward scalloping or blistering in the early appearance of the membrane. The Hertwigs in '86 observed a similar narrow perivitelline space and scalloping in ageing eggs.

Arbacia.

Some observations upon *Arbacia* eggs gave similar results, with one exception, namely, there was no initial acceleration of rate in membrane formation so characteristic of *Toxopneustes*. But this lack of acceleration may however have been due to insufficient early observations. The facts are brought together in Table II.*b*.

In experiment 1 (Table II.*b*), the eggs of 2 out of the 5 females that formed membranes at all, after the first observations, showed retardation in rate with age.

In experiment 3, the eggs of 4 out of the 7 females that formed membranes after the first observation, also showed retarded rate of membrane formation.

In experiment 2, one female showed retardation, another showed no change in rate and the other 4 did not form membranes after the first observation.

At this point the question arose whether the inability to form membranes was due to the sperm or to the egg or to both. The matter was tested as follows: *after eggs and sperm of the same age no longer formed membranes*, the eggs were fertilized by fresh sperm, and vice versa, freshly liberated eggs were tested with old sperm. In the first case, *i. e.*, stale eggs fertilized by fresh sperm, fertilization membranes reappeared, and were formed in most instances as rapidly as in previous matings. For example, in experiment 3, membranes ceased to appear when both germ cells were 23 hours old. When 24 hours old, the eggs were fertilized by 4-hour-old sperm, and membranes appeared in 3 out of 7 females. The eggs of 2 females formed membranes in 2½ minutes each, which was more rapid than when the eggs were 18½ hours old. The third formed membranes in 6 minutes. These observations showed that the first inability to form membranes was not due to the absence of membrane-forming substance in the eggs.

When fresh sperm was used with eggs 42, 48, 65 and 70 hours old, no membranes were formed. This showed that at this age the lack of membranes was now due to a change in the eggs which began about 24 hours after liberation in some females, and in 42 hours in other females. The membrane substance was gone.

In the reverse experiments, when increasingly old sperm was used to fertilize fresh eggs, membranes were formed as rapidly and as extended from the surface of the egg as when fresh sperm was used (within certain limitations). For example in experiment 4, 23-hour-old sperm caused membranes to appear in 7, 3, 3, 3 minutes in the 4 females. In experiment 5 the sperm which was 45½ hours old formed membranes in 2 of the 3 females in 2 and 2½ minutes respectively. In experiment 6, when sperm was 73 hours old, membranes appeared in 2½, 4, 4½ and 5 minutes respectively. Even when sperm was 95 hours old (experiment 6) one out of 4 females formed membranes in 2½ minutes.

These and other facts clearly show that *the rate and charac-*

ter of membrane formation, in aging eggs, are functions largely of the egg and determined by their physiologic condition at liberation and by their subsequent age. Physiologically good eggs form clear membranes rapidly with either fresh or old sperm (the maximum age of such sperm was not ascertained). Physiologically poor eggs, form membranes more slowly, closer to the egg surface or do not form membranes at all with either fresh or stale sperm. The change in rate of membrane formation with age, constitutes the third index of the physiologic deterioration of the eggs with age.

Effect of Age Upon Cleavage.

Synchronous Aging of Germ Cells.

Aging or physiologic deterioration may be measured either (1) by a change in size, or (2) a loss of the jelly layer, or (3) a change in rate and character of membrane formation. There is another, more exact and more finely graded index of the changes in the germ cell with age, namely, (4) changes in cleavage.

Hertwig, '96, and Loeb suggested that with age there was a decreasing cleavage. F. R. Lillie, '14, showed in much greater detail that there was a decrease and indicated the extent of the decrease.

In Study I., I showed that under constant and optimum conditions freshly liberated eggs from different freshly collected females frequently differed widely in the total per cent. of cleavage in a given time, even when fertilized by the same male. In *Toxopneustes* the different females vary from 11 to 87 per cent., in *Hipponoë* from 5 to 81 per cent., in *Arbacia* from 0 to 90 per cent. Such differences were ascribed to differences in physiologic condition of the eggs of the different females at the time of liberation.

In preliminary experiments, a given pair of tested sea urchins were used for successive fertilizations. From 100 to 200 of the fertilized eggs were examined at each interval, and the sperm used was freshly prepared from the dry sperm.

In one group of experiments there was an unmistakeable direct reduction in the total cleavage with age. For example, in experiment 1, the eggs of 3 females were fertilized by one male when the germ cells were from ¼ to 6 hours old. The per cent. of the total number of eggs that cleaved at each interval was 98 per cent. when 17 minutes old, 80 per cent. when 42 minutes old, 70 per cent. when 98 minutes old, 33 per cent. when 161 minutes old, 40 per cent. when 214 minutes old, 18 per cent. when 294 minutes old, 0 per cent. when 360 minutes old. The other 2 females showed corresponding decreases. The average for the 3 females was 94, 62, 54, 28, 31, 11 and 0 per cent. respectively. See Table III.

In the other group of experiments, there was an initial increased cleavage in the eggs of some or all of the females, followed by a definite and progressive decrease. For example in experiment 6, the eggs of female 1 and 4 decreased in cleavage with age, as in the first group. But the eggs of females 2 and 3 increased in cleavage for a brief period after the first observation, and then the per cent. decreased. This appeared to me at the time to be an error in observation, but similar increments occurred in other experiments. In experiment 7 the eggs were fertilized at early and frequent intervals, namely, 7, 20, 43, 59, 89 and 140 minutes. All 5 series of mating showed an early *progressive increased cleavage*, followed after 1 hour, by a decrease. The average for all 5 series was 49, 66, 88, 62, 40 and 25 per cent.

It is probable then that in the other experiments the apparent absence of an initial increased cleavage was due to failure to make sufficiently early and sufficiently frequent intervals, or possibly that the increase in cleavage becomes evident only in eggs in poor physiologic condition.

In *Arbacia* only the direct decreased cleavage was observed. Whether an initial increase occurred I cannot say. In the first place insufficient early observations were made, and in the second place, with physiologically good eggs, the maximum or nearly maximum cleavage took place, so that any physiologic change could not be manifested by an increased cleavage.

Only two experiments are given to illustrate the behavior of aging eggs of *Arbacia*. In experiment 8, the eggs of five females fertilized by one male, averaged 80 per cent. when 4 hours old, 33 per cent. when 23 hours old, 0 per cent. when 28½ hours old. (See Table III.)

TABLE III.

SHOWS CHANGE IN TOTAL CLEAVAGE WITH PROGRESSIVE AGE OF GERM CELLS.
Toxopneustes AND *Arbacia*.

Toxopnuestes.

No.	Date.	Age of Germ Cells in Minutes.	Per Cent. of Eggs that Cleaved.							Time After Fertilization, Minutes.	Average Per Cent. Cleavage.	Per Cent. Loss per Hr.
			Females 1.	2.	3.	4.	5.	6.	7.			
1	7/12	17	98	98	87					120	94	
		42	80	76	31						62	
		99	70	72	20						54	
		161	33	38	14						28	
		214	40	30	23						31	
		294	18	14	2						11	
		360	0	0	0						0	18.0
2	7/19	130	82[1]	96	83	96	98	94		120	91	
		240	93	99	84	89	93	91			91	
		350	92	98	82	64	81	12			71	
		470	77	64	70	4	82	10			51	7.0
3	7/16	160	50	74	81	96	18[1]	14	40	120	53	
		300	34	61	37	92	34	0	50		40	
		500	28	8	20	29	1	6	25		16	6.5
4	7/21	120	51	98						120	74	
		323	11	84							47	8.0
		450	0	0							0	
5	7/14	20	92	47	98					120	79	
		80	99	95	97						97	
		140	98	95	97						96	
		210	96	93	97						95	
		280	92	82	87						87	
		370	62	69	85						72	
		440	82	59	87						76	
		500	43	10	36						29	8.2
		810	0	0	0						0	
6	7/7	75	91	41	67	86				120	71	
		84	75	87	98	80					85	
		180	80	80	17	60					59	7.2
7	7/3	7	80	23	35	68	43			113	49	
		20	95	46	75	73	42			105	66	
		43	80	85	88	95	95			87	88	
		59	62	71	76	19	86			91	62	
		89	38	50	73	31	11			91	40	
		140	34	12	9	11	11			85	25	

Arbacia.

8	8/12	4 hrs.	95	97	18	95	95			60	80	
		23 "	57	49	0	0	60			67	33	2.5
		28½ "	0	0	0	0	0			60	0	
9	8/16	1½ "	100	98	100	88	98	92	100	64	96	
		18½ "	75	20	95	62	88	55	51	60	63	1.8
		23 "	0	0	0	0	0	0	0	60	0	

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[1] Probably an error in recording.

Fig. 1 represents schematically the change in rate of cleavage with age.

The per cent. cleavage is a convenient and finely graded index not only of the physiologic condition of the eggs at the time of liberation, but at subsequent intervals. It became then a simple matter to compute the rate of deterioration with age for a given female, or group of females. The rate of deterioration, *i. e.*, the reduction in cleavage, in per cent. per hour, varied from 6.5 per cent. per hour in experiment 3, to 18.0 per cent. in experiment 1, viz: 6.5, 7.0, 7.2, 8.0, 8.2 and 18 per cent. per hour, for nearly comparable periods. These rates are strikingly greater in *Toxopneustes* (from tropical waters) than in *Arbacia* (of the temperate waters of Massachusetts). Experiments 8 and 9 are illustrative of the rate of deterioration in *Arbacia*, viz: 1.8 per cent. per hour, in experiment 9, 2.5 per cent. per hour, experiment 8. When it is recalled that the difference in temperature of the waters at the two laboratories is about 10° C., and the difference in physiologic deterioration 3 to 10 times as great, it must be evident that the difference in rate is conditioned not only by temperature but by differences in HO concentration, and the egg protoplasm of the different species.

The above data did not make clear whether the decreased cleavage was due to the deterioration of the eggs or of the sperm or both sperm and eggs. The answer to these questions could be obtained in two ways: First to fertilize increasingly old eggs each time with freshly liberated and tested sperm, which would test the rate and degree of degeneration of the eggs. Secondly to fertilize freshly liberated and physiologically good eggs with fresh suspensions of increasingly old sperm, and test the degeneration of the sperm. Both series of experiments were made.

Effect of Age Upon Eggs.

Aging Eggs fertilized by Fresh Sperm. Longevity of Eggs. Toxopneustes.

In the following experiments, eggs and sperm aged synchronously until the eggs either no longer cleaved or only a very small per cent. cleaved. Then samples of the same eggs were fertilized at each subsequent interval by freshly liberated sperm. See Table IV.

In experiment 1, for example, the eggs of 4 females fertilized

TABLE IV.

SHOWS CHANGE IN CLEAVAGE WHEN PROGRESSIVELY AGING EGGS ARE FERTILIZED BY SPERM OF VARYING AGES. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.		Per Cent. of Cleavage Eggs.						Time in Minutes.	Average Cleavage Per Cent.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.		
1	7/2	64	64	0	1	0	1			120	½
		209	19	8	85	70	10				43
		250	60	24	80	50	26				45
		309	120	3	44	14	17?				19
		400	3	8	70	2	10				22
2	7/12	420	420	6	2	0				120	3
		436	60	98	92	60					83
3	7/14	500	500	43	10	36				120	29
		600	2	50	36	81					55
		720	720	0	0	0					0
		10	600	33	0	0	10				10
4	7/19a	660	660	0	0	0	0	0	0	120	0
		660	90	95	94	13	32				59
		675	105	10	50	10	12	10	7		16
5	7/19b										
	a)	610	610	0	4					120	
				0	0						
				0	4						1
	b)	640	60	80	8						
				36	50						
				70	25						45
	c)	20	600	47	33	4	23				25
	d)	30	30	18	44	22					28
6	7/16	660	660	5	5					120	5
		7	660					91			91
		670	7	69	54	78					
				78	78						
				40							
		7	7				100				100
7	6/21	660	660	0						120	0
		660	20	18							18
		660	20	13							13
		20	660	0	0						0
		20	20	0	0						0

by the one male, gave a decreasing per cent. of cleavage with age, until the germ cells were 64 minutes old, when only 0, 1, 0 and 1 per cent. of the eggs of each of the females cleaved. The eggs were allowed to age further until they were 209 minutes old, when they were fertilized by freshly liberated sperm, and cleavage rose to 8, 85, 70 and 10 per cent. respectively. This was 42½ per cent. greater than the previous observation. It was clear that the failure to cleave when 64 minutes old was due to the almost complete deterioration of the sperm. It is not clear from this experiment how much the eggs have deteriorated. For as I have shown the results obtained by sperm from different individuals may not be compared without further testing. In this experiment, when the eggs were 225 minutes old, and sperm only 35 minutes old, 32 per cent. cleaved. When the eggs were 250 minutes old and the sperm 60 minutes old, the average cleavage was 45 per cent. Even when the eggs were 400 minutes old, and fertilized by freshly liberated sperm, 22 per cent. cleaved. Hence it is clear that the decline to the zero mark when both germ cells were 64 minutes old, was due not to the death or incompatibility of the eggs, but of the sperm.

Even more striking results were obtained in other experiments. In experiment 2, for example, cleavage decreased progressively until the germ cells were 420 minutes old, and 6, 2 and 0 per cent. of the eggs cleaved. When the eggs 16 minutes later, *i. e.*, 7¼ hours old, were fertilized by sperm only 1 hour old, the cleavage was 98, 92 and 60 per cent. or an increase of 80 per cent. over the last reading. When the eggs of these same females were liberated, and immediately fertilized they averaged 80 per cent. hence it is probable that very little real deterioration of the eggs occurred during the first 7 hours after liberation.

In experiment 3, when the germ cells were both 8 hours old, the average cleavage was 29 per cent., when 12 hours old 0 per cent. When the eggs were 10 hours old and the sperm 2 minutes old, the average cleavage rose to 55 per cent.

In experiment 4, 11-hour-old germ cells gave 10 per cent. cleavage. Eleven-hour-old eggs by moderately fresh sperm gave 59 per cent. cleavage. Fifteen minutes later using the same eggs and sperm, only 16 per cent. cleaved. The marked reduction was due again to the rapid deterioration of the sperm of the second male.

In experiment 5, the eggs of 2 females were tested by 3 different males. When the germ cells were 5 hours old 1 per cent. cleaved. When tested by moderately fresh sperm, cleavage rose to 45 per cent.

In experiment 6, the increased cleavage with fresh sperm was 57 to 73 per cent.

In experiment 7, the small increase with fresh sperm, *i. e.*, only 18 per cent. and 13 per cent. respectively, was due to the poor physiologic condition of the sperm as determined by tests with fresh eggs.

In every experiment, freshly liberated sperm raised the total cleavage from 0 or nearly 0 to 30, 40, 50 and even 80 per cent. of the eggs examined. There can be little doubt but that this means that the apparent death of the eggs (when no cleavage occurred) was really due to the precocious and rapid deterioration of the sperm, with moderately little deterioration of the eggs.

Hence to determine the exact physiologic condition of the eggs at any stage in the ageing cycle, and to determine the real longevity of the eggs, it is necessary to fertilize the eggs with freshly liberated sperm at each testing, as F. R. Lillie, '14, had done for a very different purpose. For example, the eggs of 4 females of experiment 1, Table V., when ⅓ hour old, gave 95 per cent. average cleavage; when 20 hours old, tested by fresh sperm, 67 per cent.; when 23 hours old, 7 per cent.; when 24 hours old, 3 per cent.; when 25 hours old, 1 per cent.; when 46 hours old, ½ per cent.; when 48 hours old, 0 per cent. These figures give a much more exact measure of the rate of deterioration of the eggs than when synchronously aged eggs and sperm

TABLE V.

SHOWS REDUCTION IN CLEAVAGE WHEN AGEING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS TRUE LONGEVITY OF EGGS. *Toxopneustes*.

No.	Date.	Age of Germ Cells in Hours.		No. of ♂.	Female No.						Average Cleavage, Per Cent.
		♀.	♂.		1.	2.	3.	4.	5.	6.	
1	7/4	$\frac{1}{3}$	$\frac{1}{3}$	1	95						95
		20	$\frac{1}{3}$	2	50	43	95	80			67
		23	$1\frac{2}{3}$	2	0	4	0	3			2
		24	$\frac{1}{10}$	4	0	13	0	0			3
		25	1	4	0	1	2	0			1
		46	1	5	0	2	0	0			$\frac{1}{2}$
		48	$\frac{1}{10}$	6	0	0	0	0			0
2	7/5	23	$\frac{1}{10}$	1	7	0	0				1
		25	1	2	45	0	2				—
		50	$\frac{1}{10}$	1	0	0	0				0
3	7/7	46	$\frac{1}{3}$	1	6	0	1	0			$1\frac{3}{4}$
		46	$\frac{1}{4}$	2	0	0	1	0			$\frac{1}{4}$
		47	$\frac{3}{4}$	3	0	2	1	1			1
4	7/8	$\frac{1}{2}$	$\frac{1}{2}$	1					90	81	85
		23	$\frac{1}{3}$	1	0	22	43	25			22
		51	$\frac{1}{10}$	2	0	0	0	0			0

were used. There was very little deterioration or dying until the 20th hour, and then a most rapid rate of destruction until the 24th hour, and a final residual minimum extending until the 48th hour. The eggs of female 1 died at 23 hours female 4 at 24 hours, female 3 at 25 hours, female 2 at 48 hours.

Similar results were obtained in experiments 2, 3 and 4. Forty-eight hours appeared to be the maximum longevity of the eggs of *Toxopneustes* under the given experimental conditions. Table V. gives the rate of deterioration of the eggs, Table III. of the sperm.

Arbacia.

The experiments were repeated with *Arbacia* eggs, and the inquiry was pushed further. The data are brought together in Table VI. The facts may be summarized as follows:

1. Eggs which no longer cleaved when fertilized by sperm of the same age, did cleave when fertilized by freshly liberated sperm. In experiment 1, the increase was from 0 to 39 per cent.; in experiment 2, from 0 to 56 per cent.; in experiment 3, from 0 to 44 per cent., etc. This is exactly as in *Toxopneustes*.

2. With further aging there occurred a second cycle of progressively decreasing cleavage. For example in experiment 5 24-hour eggs fertilized by 1-hour sperm averaged for the 6 females 42 per cent. When the same germ cells were 31 and 8 hours respectively, they averaged only 10 per cent. A similar secondary deterioration occurred in experiment 1. The rapid deterioration was due in both groups to the more rapid destruction of the sperm than of the eggs, as in *Toxopneustes*.

3. The real deterioration of the *eggs* was determined as in *Toxopneustes* by fertilizing aging eggs with freshly liberated sperm at each testing. It was found that the eggs were very long lived. In experiment 7, 20-hour eggs showed little deterioration, 96 per cent. cleaved. Even when 41 hours, there was little deterioration, for 81 per cent. cleaved. When 48 hours 19 per cent., when 65 hours 9 per cent., when 72 hours 1 per cent. The other experiments, viz: 2, 3, 5, 6, 7, 8, 9, 10, 11, etc., gave similar results. They showed the same characteristics as *Toxopneustes* eggs, namely, *a long period of little deterioration, which in Arbacia is about 20 hour under the given experimental*

TABLE VI.

SHOWS REDUCTION IN CLEAVAGE WHEN AGING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS LONGEVITY OF EGGS OF *Arbacia.*

No.	Date.	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀.	♂.	1.	2.	3.	4.	5.	6.	7.			
1	8/12a	4	4								60	80	
		$28\frac{1}{2}$	$28\frac{1}{2}$	0	0	0	0				60	0	
		$28\frac{1}{2}$	$4\frac{1}{2}$	29	33	28	67				60	39	
		48	$\frac{1}{2}$	2	$\frac{1}{2}$	0	0				60	1	1.8
		$1\frac{1}{2}$	$1\frac{1}{2}$								60	96	
2	8/16	23	23	0	0	0	0	0	0	0	60	0	
		24	4	81	79	82	0	65	82	8	60	56	
		42	1	30	0	80	0	0	0	0	90	15	
		42	$\frac{1}{6}$	77	0	72	0	0	0	0	60	21	
		48	$\frac{1}{10}$	90	3	79	0	0	0	0	90	24	
		65	$\frac{3}{4}$	48	0	0	0	0	0	0	60	7	
		70	$\frac{1}{12}$	21	0	0	0	0	0	0	60	3	1.3
		88	$\frac{1}{12}$	0	0	0	0	0	0	0	60	0	
3	8/17	$1\frac{1}{2}$	$1\frac{1}{2}$	96	70	97					120	87	
		$18\frac{1}{2}$	$18\frac{1}{2}$	71	20	93						61	
		23	23	0	0	0						0	
		24	4	28	50	65						44	
		42	1	30	0	17						15	
		42	$\frac{1}{10}$	66	0	70						45	
		48	$\frac{1}{0}$	52	3	74						43	
		65	$\frac{1}{4}$	45	4	1						16	
		70	$\frac{1}{12}$	18	6	5						9	1.1
4	8/17	46	$\frac{1}{12}$	40	41	67					60	43	
		7	25	0	0	0						0	
5	8/13	4	5	98	97	99	99	98	93		120	97	
		24	1	87	3	57	89	19	0			42	
		31	8	64	0	0	0	0	0			10	3.2
		49	$1\frac{1}{4}$	0	0	0	0	0	0			0	
		49	$1\frac{1}{4}$	0	0	0	0	0	0			0	
6	7/29	20	$\frac{1}{12}$	73	85						120	79	
		43		34	35							34	
		51		7	5							6	2.3
7	8/1	20–22	$\frac{1}{12}$	96	96						120	96	
		41		75	87							81	
		48		14	24							19	
		65		3	15							9	
		72		2									1.8
8	8/3	41	$\frac{1}{12}$	11	22						120	16	
		63		8								8	
		87		0								0	
9	8/9	48	$\frac{1}{12}$	9	36						120	22	
		68		0								0	
10	8/10	45	$\frac{1}{12}$	2	7						120	4	
		49		1	7							4	
		51		0	0							0	
		69		0	0							0	
11	8/11	20	$\frac{1}{12}$	89								89	
		40		7								7	

* Much cleavage, exact count not made.

Table VI.—*Continued.*

No.	Date	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀4.	♂4.	1.	2.	3.	4.	5.	6.	7.			
12	8/18	6	28	49	99	95	99				90	85	
		1	$45\frac{1}{2}$	0	0	0					60		
				91	47	97					120	81	
		5	$49\frac{1}{2}$	0	17	85					60		
				93	31	77					120	67	
		$\frac{1}{10}$	68	39	90	52	48				60		
				100	100	83	40				120	80	
		5	73	66	74	75	45				60		
				90	69	87	100				120	86	
		10	77	*	*	*							
		$\frac{1}{10}$	95	29	59	72	78				275	59	
13	8/12a	48	48	14							60		
				14							120	14	
	b	18	48	80							60		
				98							120	98	
	c	$\frac{1}{12}$	48	60							60		
				70							120	70	
	d	48	18	11							60		
				13							120	13	
	e	18	18	89							60		
				92							120	92	
	f	$\frac{1}{12}$	18	58							60		
				70							120	70	

conditions. Then there occurred a period of rapid deterioration, i. e., until the 40th hour, and thirdly a long period of minimum cleavage ranging from 50th to the 70th hour. *Arbacia* differs only in the rate of deterioration, which is several times slower than in *Toxopneustes.*

4. The longevity of *Arbacia* eggs was correspondingly longer than *Toxopneustes.* In experiment 1, the eggs of 2 out of 4 females ceased to cleave when 48 hours old. In experiment 2, the eggs of 4 females ceased to cleave at 42 hours; the fifth female at 65 hours, and the 6th female at 88 hours. In experiment 3, cleavage occurred in all 3 females at least till the 70th hour, etc., etc. The longevity ranged from 24 hours to beyond 72 hours, or about twice that of *Toxopneustes* eggs, due largely to the difference in temperature (about 10° C.) of the sea water.

EFFECT OF AGE UPON SPERM.

Freshly Liberated Eggs Fertilized by Aging Sperm. Longevity of Sperm.

In the reciprocal experiments, after the synchronously aging germ cells, no longer cleaved, the aging sperm were tested against freshly liberated eggs.

In experiment 3 (Table IV.), when the *Toxopneustes* germ cells were both 8 hours old, 29 per cent. of the eggs cleaved; when 12 hours old 0 per cent. cleaved. When the sperm were 10 hours old, they were tested by fresh eggs, 10 minutes old, and gave an average cleavage of 10 per cent. This might suggest that the rate of deterioration in synchronously aging sperm cells was conditioned by the rate of deterioration of the sperm.

In experiment 5, 10–hour–old eggs fertilized by 10-hour-old sperm averaged 1 per cent. cleavage. When the sperm was tested by fresh eggs, the cleavage rose to 25 per cent.

Similarly in experiment 6, 11-hour old germ cells averaged but 5 per cent. cleavage; the old sperm by fresh eggs gave 91 per cent. cleavage. On the other hand, in experiment 7, 11-hour-old germ cells gave 0 per cent. cleavage, in both crosses.

The explanation for these apparently contradictory data is found in a triple comparison, namely,

1. Crossing of synchronously aged germ cells.
2. Crossing of aged eggs by fresh sperm.
3. Crossing of fresh eggs by aging sperm.

If this be done it becomes apparent at once that the results are due in largest part to the condition of the egg, subject only to the ability of the sperm no matter how deteriorated to penetrate the cortical layer and initiate development.

In experiment 5, Table IV., 10-hour-old germ cells gave 1 per cent. average cleavage. When the eggs 10½ hours old were fertilized by moderately fresh sperm, 45 per cent. cleaved; the reciprocal cross, viz,, fresh eggs by the old sperm, caused only 25 per cent. to cleave. When these fresh eggs were tested by fresh sperm, 28 per cent. cleaved, *i. e., approximately the same cleavage as when fertilized by 10-hour-old sperm.* Hence low cleavage in experiment *a* was due to the sperm, while in *c* and *d* it was due to the eggs.

In experiment 6, similar results were obtained. Eleven-hour-old germ cells gave 5 per cent. cleavage. Fresh eggs used to test the old sperm gave *91* per cent. cleavage. But when these fresh eggs were tested by fresh sperm, *approximately the same percentage cleaved as when fertilized by stale sperm.*

Experiment 7 is interesting because the fresh eggs used were in physiologically poor condition, and when fertilized by 11-hour-old sperm gave 0 per cent. cleavage. These same eggs tested by freshly liberated sperm gave the same results, namely 0 per cent.

Hence the results obtained by fertilizing fresh eggs by old sperm is determined in largest part by the physiologic condition of the eggs. The sperm plays but a minor rôle. One must be cautious, however, in any given experiment, in interpretng the results. In experiment 3, for example, the 10-hour-old sperm gave only 10 per cent. cleavage with fresh eggs. This low cleavage might have been due to the physiologically poor condition of the sperm or of the eggs. Actual tests showed that the fresh eggs were in poor condition and not the old sperm.

If the sperm deterioration is not reflected, or only to a very slight degree reflected, in a reduction in cleavage how does it happen that in all experiments *when aged eggs were fertilized by aged sperm the cleavage was far less than in either of the other two crosses.*

This result is probably due to a summation of effects, that due to the deteriorated condition of the eggs, and that due to the probable greater difficulty of aged sperm to initiate mitosis and cleavage. In the final analysis both are reducible to the condition of the egg.

Arbacia.

Further light was thrown upon these questions by the experiments with *Arbacia*.

Experiment 12, Table VI., is illustrative of the effect of aging upon the sperm. In this experiment after the germ cells no longer cleaved, the old sperm was tested against fresh eggs of 4 different females, over a long range of intervals. When the sperm were 28 hours old, the eggs of the 4 females averaged 85 per cent. cleavage (90 minutes after fertilization). When the sperm were 45½ hours old 81 per cent. of the fresh eggs of 4 other females cleaved; *when 73 hours old 86 per cent.; when 77 hours old "very many" (exact count not taken); when 95 hours old 59 per cent. of the eggs cleaved.* At this last observation the different females gave 29, 59, 72 and 78 per cent. cleavage. How much longer the sperm could have fertilized freshly liberated eggs was not determined. How much of the reduction (when the sperm were 95 hours old) was due to a physiologic deterioration of the sperm, comparable with that in the eggs, and how much the reduction was due to an excessive mortality, as a result of which there were insufficient numbers of effective sperm, was not determined. Both factors probably play important rôles, though I am inclined to believe that a differential mortality plays a major rôle.

In the above experiment and in others, *the decrease in cleavage with these very old sperm was amazingly small, far less than in eggs of like age.* It should be noted however that the sperm in these experiments were allowed to age in the "dry" or concentrated condition, without the addition of sea water, and in this condition their metabolism is at a minimum, hence the maximum longevity.

In experiment 13, various types of crosses corroborate the above conclusions. Eggs and sperm were fertilized at varying intervals until both germ cells were 48 hours old, at which time only 14 per cent. cleaved. This 48-hour-old-sperm was tested with 18-hour-old eggs and with $\frac{1}{12}$-hour-old eggs. The 18-hour eggs gave 98 per cent. cleavage; the $\frac{1}{12}$-hour eggs 70 per cent. Samples of the same eggs were also fertilized by sperm 18 hours old. The 48-hour eggs gave 13 per cent. cleavage; the 18-hour eggs 92 per cent. and the $\frac{1}{12}$-hour eggs 70 per cent. It will be at once evident that the sperm at both ages gave a *remarkably similar cleavage per cent. with fresh, with moderately old and with old eggs*, *i. e.*, the sperm have undergone little if any physiologic deterioration between 18 and 48 hours after liberation. The 48-hour eggs gave 14 per cent. cleavage by 48-hour sperm and 13 per cent. with 18-hour-old sperm. The 18-hour-old eggs gave 98 per cent. cleavage with 48-hour-old sperm and 92 per cent. with 18-hour sperm. The $\frac{1}{12}$-hour-old eggs gave 70 per cent. cleavage with 48-hour-old sperm and the same per cent. with 18-hour-old sperm.

The cleavage was determined essentially or exclusively by the condition of the eggs.

The decrease in cleavage in the fresh eggs was due as in *Toxopneustes* experiment to the poorer physiologic condition of the eggs at the time of liberation. *Good fresh sperm cannot cause physiologically poor eggs to cleave to any greater extent than old* *sperm. Cleavage in physiologically good eggs is not decreased by old sperm.*

From these data it will also be observed that the longevity of dry sperm was greater than the eggs in the given experimental conditions. In neither *Toxopneustes* nor in *Arbacia* was the maximum longevity of the sperm determined.

Effect of Age Upon Rate of Cleavage.

I have shown that the total number of eggs that cleaved in a given time after fertilization, decreased as the eggs aged. I propose now to show that there was, as F. R. Lillie, '14, indicated, a corresponding retardation of the rate of early cleavage, with increasing age of the egg; and in a later paper I propose to describe the irregular and abnormal character of cleavage in very aged eggs.

The rate of cleavage was ascertained by the total cleavage at three intervals, namely, (1) 40 minutes after fertilization, when the eggs first divided, (2) 1 hour after fertilization, when most and sometimes all the eggs had divided once, and (3) 2 hours after fertilization when all or very nearly all the eggs had divided at least once.

The data are brought together in Table VII.

In experiment 1, the eggs of 6 females were fertilized by one male, when the germ cells were 130, 240, 350 and 470 minutes old. A record was made at each interval, of the number of eggs that cleaved in 40, 60 and 120 minutes after each fertilization. In female no. 1, 54 per cent. cleaved within 40 minutes, when the germ cells were 130 minutes old; 37 per cent. cleaved in the same interval when 240 minutes old; 3 per cent. when 350 minutes old, 5 per cent. when 470 minutes old. *There was an undoubted decrease in the rate of first cleavage with age. The cleavage at the same ages 60 minutes after fertilization also showed a similar retardation with age of the germ cells*, for 78, 85, 51 and 59 per cent. respectively, cleaved. The total cleavage, namely 2 hours after fertilization, as I have shown before, also decreased with age. The corresponding figures were 82, 93, 92 and 77 per cent.

The average for all 6 females brings out forcibly the retardation and decrease in cleavage. The average for the germ cells 40

TABLE VII.

SHOWS CHANGE IN RATE OF CLEAVAGE WITH AGE OF GERM CELLS. THE FIGURES REPRESENT THE PER CENT. CLEAVAGE IN INDICATED TIME. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Female 1.			2.			3.			4.			5.			6.			Average Per Cent			Ratio of Cleav in 40 Min.: 120 Min.	Ratio of Cleav in 60 Min.: 120 Min.
			40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.		
1	7/19	130	54	78	82	70	93	96	73	88	83	82	88	96	86	94	98	91	80	94	76	86	91	83	94
		240	37	85	93	83	99	99	46	56	84	67	76	89	78	78	93	82	67	91	65	76	91	71	83
		350	3	51	92	72	94	98	22	59	82	67	57	64	70	75	89	35	26	12	44	60	71	62	84
		470	5	59	77	17	46	64	16	43	70	0	0	4	64	75	82	1	6	80	27	39	51	53	76
2	7/12	17		98			98			87															
		42	56	77	80	34	78	76	18	30	31										36	62	62	58	100
		99	0	35	70	6	50	72	1	12	20										2	32	54	3	59
		161	4	15	33	6	18	38	1	11	14										3	14	28	10	50
		214	3	24	40	6	28	30	1	11	13										3	21	31	9	67
		294	3	14	18	½	17	14	0	0	2										1	10	11	9	90
		360	0	0	0	0	0	0	0	0	0										0	0	0	0	0
		420	0	0	0	0	0	0	0	0	0										0	0	0	0	0
3	7/14	20	½	93	92	0	32	61	17	98	98										5	74	83	6	89
		80	0	87	99	0	95	95	0	35	70										0	72	88	0	81
		140	2	91	98	2	95	95	2	99	97										1	95	96	1	98
		210	8	83	96	8	63	93	12	97	97										9	81	95	9	85
		280	6	92	92	32	77	82	5	86	87										14	85	87	16	97
		370	6	61	62	4	46	69	3	47	85										4	51	65	6	78
		440	0	25	62	0	20	59	0	51	87										0	32	69	0	46
		500	0	12	43	0	½	10	0	5	36										0	6	29	0	20
4	7/16	160	0	83[1]	50	0	74	74	0	26	81	0	28	96	0	20	18				0	38	53	0	
		300	0	39	34	0	60	61	0	54	37	0	34	92	0	38	34				0	45	49	0	
		500	3		28	0		8	5		20	4		29	1		66				2		26	7	

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[1] Probably an error in recording.

minutes after fertilization was *76, 65, 44 and 27 per cent.* for the 4 different ages of the germ cells. The average cleavage 60 minutes after fertilization, for the same ages, was *86, 76, 60 and 39 per cent.* and the total cleavage, *i. e.*, 2 hours after fertilization, was 91, 91, 71 and 51 per cent. respectively.

These observations suggest an explanation for the decreasing total cleavage with aging germ cells. For the decreased total may conceivably be due to a retardation of the division process, or an inhibition of this process, or both.

Records taken three hours after fertilization showed that the 2-hour observation really was the total cleavage; that very little and usually no additional cleavage occurred. *Hence the decreased total cleavage with aging germ cells was not an apparent decrease due to retardation, but a real decrease by inhibition of cleavage.*

We are concerned then with two symptoms or independent evidences of changes in the eggs caused by their aging. On the one hand, an increasing number of eggs were prevented from developing and, on the other hand, those that do develop, do so with increasing difficulty (retardation).

The relation between retardation and reduction in cleavage may be expressed as follows: When germ cells were 130 minutes old, 83 per cent. of the total number of dividing eggs cleaved within 40 minutes after fertilization; when the germ cells were 240 minutes old a less number, namely 71 per cent. of the cleaving eggs, divided in the same time. When the germ cells were 350 and 470 minutes old, 62 and 53 per cent. of the segmented eggs cleaved in 40 minutes.

In experiment 2, germ cells in poor condition were tested at 8 intervals, between ¼ hour and 7 hours, and the rate of cleavage noted at each interval. The results are essentially in accord with those of experiment 1. The per cent. cleaving in 40 minutes after each fertilization was 36, 2, 3, 3, 1, 0, 0 per cent.; for 60 minutes after fertilization, the record was 62, 32, 14, 21, 10, 0, 0; and the total cleavage 2 hours after fertilization was 62, 54, 28, 31, 11, 0, 0 respectively.

Experiment 4 is also quite in accord with the previous ones.

Experiment 3 differs from the above only in the increasing total cleavage for a brief period, followed by decreasing cleavage with further age. The rate of cleavage follows a similar cycle; *the rate of cleavage and the total cleavage increased with the age of the germ cells until they were 280 minutes old, after which the rate was retarded and the total cleavage declined.*

Arbacia.

With *Arbacia* eggs the rate of cleavage was ascertained by noting the per cent. of the eggs that reached the 4-cell stage in 60 minutes. The data are shown in Table VIII.

TABLE VIII.

SHOWS CHANGE IN RATE OF CLEAVAGE FROM THE PER CENT. OF EGGS THAT REACH THE 4-CELL STAGE IN 60 MINUTES. *Arbacia.*

No.	Date.	Age of Germ Cells in Hours.		Female 1.	2.	3.	4.	5.	6.	7.	Time, Minutes.
		♀.	♂.								
1	8/12	4	4	24	0	0	0	3			60
		23	23	1	0	0	0	1			67
		28	$4\frac{1}{2}$	2	6	5	12	36			67
		24	1	1	0	0	18	1			
		$30\frac{1}{2}$	8	0	0	0	0	0			
2	8/16	$1\frac{1}{2}$	$1\frac{1}{2}$	80	81	90	30	80	65	93	60
		$18\frac{1}{2}$	$18\frac{1}{2}$	3	0	0	0	0	0	1	
		24	4	3	10	1	0	6	1	1	
		42	$\frac{1}{6}$	1	0	0	0	0	0	0	
		48	$\frac{1}{8}$	3	0	2	0	0	0	0	
		65	$\frac{3}{4}$	4	0	0	0	0	0	0	
		70	$\frac{1}{10}$	2	0	0	0	0	0	0	
		88	$\frac{1}{10}$	0	0	0	0	0	0	0	
3	8/14b	$1\frac{1}{2}$	$1\frac{1}{2}$	42	22	67	77	90			60
		5	5	35	3	88	26	40			
4	8/14a	4	5	0	1	0	19	36			60
5	8/18	1	23	0	1	0	0				87
		5	49	0	8	0					60
		$\frac{1}{10}$	68	0	6	0					60
		5	73	5	5	3					60

In *Arbacia* as in *Toxopneustes* the total cleavage decreased and the early division process was increasingly retarded with increasing age of the germ cells. In experiment 3, for example, the eggs of 5 females were tested when their germ cells were $1\frac{1}{2}$ hours old, and 59 per cent. of the eggs reached the 4-cell stage in 1 hour. When the germ cells were 5 hours old only 38 per cent. reached the 4-cell stage in the same time. In experiment 1 and 2, older eggs were used. The results were even more striking and in entire accord with the preceding experiments.

Retardation is another symptom of the deterioration not of the sperm but of the eggs.

CORRELATION OF CHANGES.

I have shown firstly that the freshly liberated eggs of freshly collected females of the same species varied considerably with respect to size, jelly layer, membrane formation and cleavage; secondly the range of variability for each of these types of changes was ascertained; thirdly, these changes were correlated; fourthly, by *these correlated phenomena one could accurately and conveniently ascertain the exact physiologic condition of the eggs of any female, at any time.*

Freshly liberated eggs from freshly collected females could be readily classified into physiologically "good," "poor" and "bad" eggs, on the basis of the correlated phenomena, as follows:

	Physiologically Good Eggs.	Physiologically Poor Eggs.	Physiologically Bad Eggs.
Size:	Norm for species. Small deviation from norm.	Larger than norm. Increasing deviation.	Still larger or smaller than norm. Still further deviation.
Jelly layer:	Maximum number with jelly layers 100–90 per cent.	Increasing loss of layer 96–70 per cent.	Further loss 70–40 per cent.
Membrane formation:	Formed within 2 minutes.	Increasingly retarded. 3–7 min.	None.
Cleavage:	Maximum number cleave 100–90 per cent.	Decreasing number 90–60 per cent	Further decrease. 60–0.

Perhaps one example chosen at random may make the matter more definite. The freshly liberated eggs of *Arbacia* (Experiment 8–12) were practically normal in size, over 90 per cent. contained the jelly layer, fertilization membranes were formed in 1½ to 2 minutes, the cleavage was 95, 97, 96 and 94 per cent. in the eggs of 4 out of 5 females. The eggs of the fifth female were larger than the norm, less than 90 per cent. possessed jelly layers, no membranes were formed upon fertilization and only 18 per cent. cleaved. The eggs of the same females were tested by a second male, and gave the same size and jelly count, as before, and membranes appeared in 2, 2, 2½, 2½ minutes respectively. The cleavage count was 72, 69, 62 and 55 per cent. This retardation in membrane formation and reduction in cleavage was clearly due to the poor physiologic condition of the sperm. The eggs of the fifth female were likewise fertilized by this male and showed the same size and jelly count as before, but no membrane appeared and only 4 per cent. cleaved. By these tests it was definitely determined that the eggs of the first four females were in good physiologic condition, those of the fifth female in poor condition.

By these tests it is possible to state accurately whether any sample of eggs are "good," "poor" or "bad," and to state exactly to what degree of physiologic deterioration such sample of eggs may have reached. The words "good," "poor" or "bad" now have a specific meaning, in terms of definite measurable changes in size, loss of jelly, rate of membrane formation and cleavage, which changes symptomize and measure definite physiologic and morphologic changes in the eggs.

The freshly liberated eggs whose physiologic condition was determined, subsequently changed or aged or overripened, with respect to the same categories, namely, size, jelly layer, membrane and cleavage.

I have shown in this paper exactly the extent to which each of these categories varied with the aging of the germ cells. I wish now to emphasize the fact that the changes are correlated.

These correlated changes, each of which measures the degree of physiologic deterioration with age, may be briefly summarized in the following table:

	Freshly Liberated or Good Eggs.	Moderately Stale or Aged Eggs.	Very Stale or Aged Eggs.
Size:	Little deviation from the norm.	Increasing deviation in a plus direction.	Further deviation in plus or minus direction.
Jelly layer:	Maximum or close to maximum.	Decreasing.	Further decrease.
Fertilization membrane:	Within 2 minutes. Wide membranes.	Increasingly retarded. Increasingly narrow.	None.
Cleavage:	Maximum rate. Maximum number.	Increasing retardation. Decreasing numbers.	Further retardation. Further decrease.

Whatever the physiologic condition of the eggs when liberated, they undergo with age an increase in size, a decrease in the number possessing the jelly layer, a decrease in rate of membrane formation, and a decrease in cleavage, varying in degree with the condition of the eggs at the time of liberation. And one may predict the extent of the other changes from any known one or two. If size or membrane rate are known, one may predict very approximately the other symptoms of the physiologic condition of the eggs, such as jelly and cleavage, etc.

The discussion of these results will be postponed until further data will be presented, concerning other types of changes in very aged eggs. For the present I wish to draw attention to the following considerations.

1. The aging process or processes which are symptomized in the various changes described in this paper begin not with the liberation of the eggs, but upon their maturation, within the body of the mother. This was first suggested by Loeb, and I am in entire accord with his view. Hence it follows that chronologic age (time since liberation) affords but a poor idea of the real physiologic condition of the eggs either at liberation or at any interval thereafter.

2. Aging is a continuous process, beginning within the body and continuing (with somewhat accelerated rate) outside of the body, culminating ultimately in the death of the eggs.

3. Aging was manifested in a number of ways, any one of which may serve as an index of the physiologic condition, and the degree of deterioration. *The ensemble of the various indices forms a clear and unmistakeable measure of their condition and their deterioration.*

4. While no attempt has thus far been made to describe the nature of the chemico-physical processes involved in the aging of the eggs, they may nevertheless be accurately measured. It is now possible to measure very accurately the physiologic condition of the eggs at liberation, and at any interval of time thereafter, to measure accurately the rate of deterioration or senescence under given experimental conditions. It is also possible to measure the real longevity of the eggs or of the sperm.

5. These data, and those described in Part III., afford a basis for an understanding of the nature of the aging process, and of the means of controlling senescence of the germ cells.

The discussion of the results and a more complete bibliography are given in Part III.

SUMMARY.

The variation in size of the eggs, the per cent. with jelly layers, the rate of membrane formation and the total cleavage were ascertained for large numbers of freshly liberated eggs from freshly collected females, examined at different periods of the breeding season.

Three species of sea urchins were studied in this way, namely, *Toxopneustes* and *Hipponoë* of tropical waters, and *Arbacia* of the North Atlantic.

Large variations from the norm were observed and measured in all four categories. These variations were interpreted as indices of the physiologic condition of the eggs of each female at the time of liberation.

With the physiologic condition of freshly liberated eggs of a given female known, experiments were then instituted to ascertain the nature and extent of the changes in the germ cells, as they aged, or became overripe, under given optimum laboratory conditions.

The following is a brief statement of the changes in such aging germ cells, viz., changes in size, jelly layer, membrane formation and cleavage.

Change in Size.

Freshly liberated eggs in good physiologic condition varied but slightly from the norm. For details see text.

As these eggs aged, their volume increased continuously, until they cytolized or fragmented, and became smaller than the norm.

Freshly liberated eggs in poor physiologic condition either enlarged but little with age, or were directly reduced in size by cytolysis or fragmentation.

The nature, extent, and rate of change in size, depends upon the physiologic condition of the eggs when freshly liberated. Whatever the physiologic condition of the eggs may be, their senescence or physiologic deterioration can be very accurately measured by the degree of enlargement or reduction in their size.

Essentially the same results were obtained in the other two species.

Change in Jelly Layer.

Practically all freshly liberated eggs in good physiologic condition possess a jelly layer. Those in poor condition have a correspondingly less per cent.

With age the jelly layer was lost in an increasing number of the eggs.

The rate of loss depended upon the condition of the eggs at the time of liberation (all other conditions remaining constant).

For fresh eggs in good physiologic condition, the rate of loss per hour was 0.81 and for equally fresh eggs in poor condition 2.65 or over 3 times as rapid.

Essentially similar results were obtained in all three species.

The loss of jelly layer was a second symptom and index of the extent and the rate of ageing or senescence of the eggs.

Change in Membrane.

Freshly liberated eggs in good physiologic condition formed fertilization membranes within two minutes. The rate depended partly upon the sperm but primarily upon the physiologic condition of the eggs.

With increasing age the time required to form the fertilization membranes was at first accelerated and later retarded. In very aged eggs no membranes were formed.

Freshly liberated eggs in poor physiologic condition showed direct retardation in the rate of membrane formation.

Aging eggs which no longer formed membranes when fertilized by old sperm could be made to form membranes with fresh sperm.

The rate of membrane formation is practically independent of the sperm. It is essentially determined by the condition of the egg.

As the eggs aged the membrane appeared closer and closer to the surface of the egg; it became thinner and ultimately none was formed.

These observations are essentially the same for all three species.

The rate and character of membrane formation affords a third means of measuring senescence in eggs.

Change in Cleavage.

When freshly liberated eggs of *Hipponoë* and *Toxopneustes* in good physiologic condition were fertilized under the given optimum conditions at successive ages the total cleavage increased for a time and subsequently decreased,[1] and in extreme ageing none of the eggs segmented.

Cleavage then is an additional index of the degree of senescence or physiologic deterioration of the eggs.

There were two series of experiments, one in which the eggs and sperm aged synchronously, and the other in which they aged asynchronously. In both series the rate of decrease in cleavage with age was several times greater in *Toxopneustes* and *Hipponoë* than in *Arbacia*. This difference in rate of senescence as in rate of membrane formation, loss of jelly and change in size is due largely to differences in temperature, as well as to differences in HO concentration of the sea water, and to protoplasmic differences of the eggs.

When both germ cells aged synchronously the apparent longevity was about 11 hours for *Toxopneustes* and about 28 hours in *Arbacia*.

In asynchronous matings a more definite idea was obtained of the changes in the egg alone and in the sperm alone. When freshly liberated sperm were used to fertilize the eggs (of a female) at varying ages, with the precautions indicated in the text, the eggs showed progressively decreasing per cent. of cleavage (in *Arbacia*), but the rate of decrease was very much slower than when both germ cells aged synchronously. The decrease may be divided into three periods, the first a period of small decrease (about the first 20 hours in *Arbacia*) the second, a period of rapid and large decrease (between the 20th and 40th hour), and the third, a period of small decrease (between the 40th and 80th hour in *Arbacia*).

Eggs in poor physiologic condition at the time of liberation deteriorated at a correspondingly greater rate than physiologically good eggs.

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[1] Freshly liberated eggs, of freshly collected *Arbacia*, in good physiologic condition, gave a maximum or nearly maximum cleavage and with ageing there was a direct decreasing total.

The real longevity of the eggs depended upon the physiologic condition at time of liberation, the better, the longer lived, and vice versa.

The rate of cleavage was accelerated for a time and then retarded with age, more so in physiologically poor eggs, less in good eggs.

When ageing sperm fertilized freshly liberated eggs the decrease in cleavage was surprisingly small. Very little decrease occurred when sperm were 73 hours, only a little more when sperm were 95 hours old.

The explanation for the greater longevity of the sperm is found in the fact that the sperm aged in the "dry" condition, in which they are inactive and hence minimum metabolism.

The greater decrease in cleavage in synchronously ageing germ cells is due to a summation of the injurious effects upon the sperm and upon the eggs.

In very late stages in ageing of sperm, the reduction may be due not so much to physiologic deterioration as to insufficient numbers of sperm. There is ground for belief that no matter how old or deteriorated the sperm if they are active (alive) they can penetrate the egg. It is not so clear whether such aged sperm cause parthenogenesis or sexual development.

The change in size, jelly, membrane and cleavage with aging of germ cells, are accurate, convenient and corroborative indices of chemico-physical and morphologic changes in the egg as they age, and afford convenient measures of the loss in vitality, or physical deterioration. And one change may serve for this purpose. Their ensemble is convincing.

In the next study will be considered the changes in much older germ cells leading towards their cytolysis and death.

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